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THE UTILIZATION OF THE CALCIUM OF CARROTS BY ADULTS [1]

HERTA BREITER, ROSALIND MILLS, ESTHER RUTHERFORD,[2] WILLIAMINA ARMSTONG AND JULIA OUTHOUSE

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(Received for publication August 4, 1941)

INTRODUCTION

Although vegetables, because of their mineral content, have been recognized these many years as a valuable adjunct to man's dietary, only recently has it been pointed out (Mitchell and Curzon, '39) that, on a dry matter basis, some of the commonly-used vegetables are superior to milk as a source of calcium. In view of the current trend toward a more widespread and generous usage of vegetables, the question arises as to the extent to which these foods can satisfy man's need for calcium. Blatherwick and Long ('22) tried to find the answer to this question by using a mixture of vegetables; likewise, Sherman and Hawley ('22), in their attempt to determine whether or not "children could utilize calcium in the form of vegetables as efficiently as they had utilized the calcium of milk," fed more than one vegetable simultaneously. The purpose of the present work, however, was to obtain a quantitative measure of the availability of the calcium of a specific vegetable, since any data obtained on a mixture would be applicable only to a combination of those constituents in their original proportions.

[1] These data were reported, in brief, before the American Institute of Nutrition at the annual meeting in New Orleans on March 13, 1940.

[2] A portion of these data was submitted by Miss Esther J. Rutherford in partial fulfillment of the requirements for the degree of Master of Science at the University of Illinois, June, 1940.

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In the selection of the vegetable to be studied, consideration was given to native foods which were commonly and frequently used and which were readily available, from the standpoint of both supply and cost, throughout the year. Accordingly, the carrot was chosen since it fulfilled all these qualifications and enjoyed the added characteristic of being adaptable to various ways of serving. Moreover, the carrot was one of the foods comprising the basal dietary used in earlier studies (Kinsman, Sheldon, Jensen, Bernds, Outhouse and Mitchell, '39) from which the authors concluded that children on low calcium diets do not exhibit a maintenance requirement for calcium. This postulation, it was believed, could be valid only if the calcium of the basal food were no better utilized than the milk calcium. Naturally, any information on the availability of the calcium of the various constituents of that dietary would aid in settling the question.

EXPERIMENTAL

The experiment reported here is part of an investigation of the ability of seven adults to utilize dietary calcium; the first paper concerning that research dealt with milk calcium (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41). The basal period (metabolic periods 8 through 13) of the milk study also served as the basal period for the present study; it followed the period during which carrots were ingested (metabolic periods 1 through 7) and was preceded by a 7-day interval during which the subjects ingested large quantities of calcium in an otherwise unrestricted dietary. Throughout both the basal and the carrot periods, the basal dietary and the vitamin-carrying supplements were identical with those fed in the study on milk.

During the period in which the carrot calcium was being tested, all subjects received equal quantities of carrots;[3] i.e.,

[3] California-grown carrots, because of their higher calcium content and their superior palatability, were used instead of the local varieties which were already in winter storage; 400 gm., either cooked or raw, were fed. The remaining 300 gm. were consumed in the form of an unadulterated pulp-juice mixture (S & W brand) which had the same calcium content (i.e., 29 mg. per 100 gm.) as did the raw young carrot.

700 gm. daily. Such rations of a bulky food may seem unduly large, but they contained just enough calcium to give a reliable test in this type of procedure. Although the carrots furnished 202 mg. of calcium, the difference between the average calcium intakes during the carrot and basal periods was only 179 mg.; this discrepancy was due to an augmentation of the intake of basal calcium by an unanticipated increase in the consumption of bread following the withdrawal of the carrots.

RESULTS

The subjects were in good condition, both physically and mentally, throughout the experiment. Even though a temporary abdominal distress followed the ingestion of the large quantities of raw or cooked carrots, this discomfort passed off within a few hours, and all subjects had good appetites for the succeeding meal. The large amount of bulk resulted in a greater volume of fecal material and an easier evacuation of the intestine, but the time required for the passage of the carmine (which was given for the purpose of marking off the periods) was not uniformly affected; it was shortened for one subject, lengthened for four, and unchanged for the other two. A yellow pigmentation of the skin, which gradually increased in intensity and persisted for several weeks after the carrots had been withdrawn from the dietary, was noted early in the experiment. All subjects maintained their customary body weights throughout the study.

The data obtained during the carrot period are recorded in table 1 as the average daily values involved in the exchange of calcium. Inasmuch as the data for the basal period have already been reported (Breiter et al., '41), they will not be repeated in detail here. During the 35 days that carrots were eaten, the mean calcium intakes averaged 449 mg., and the daily net losses of calcium by subjects Jd, Bm, Hb, Mh, Jo, Ws and Rd, respectively, were 34, 72, 58, 74, 58, 49 and 121 mg.

During the basal period the amount of calcium ingested by the subjects averaged 270 mg., and the means of the negative balances were 67, 88, 80, 67, 120, 64 and 141 mg. in the above

TABLE 1

Daily calcium exchange and the utilization of carrot calcium.

PERIOD	INTAKE	OUTPUT		BALANCE
		Urinary	Fecal	
	Subject Jd		Weight in kg. 53	
	Age 22	Sex F	Height in cm. 166	
	mg.	*mg.*	*mg.*	*mg.*
Carrot 1	376	198	240	—62
2	429	194	276	—41
3	446	225	285	—64
4	464	252	231	—19
5	484	235	238	+11
6	433	211	238	—16
7	419	218	251	—50
Ave.	436	219	251	—34
Basal ave.	248	179	136	—67

$$\% \text{ utilization } \frac{67-34}{436-248} \times 100 = 17.6$$

	Subject Hb		Weight in kg. 60	
	Age 27	Sex F	Height in cm. 161	
Carrot 1	388	134	323	—69
2	437	155	343	—61
3	456	151	371	—66
4	474	128	416	—70
5	494	143	395	—44
6	458	155	335	—32
7	434	154	343	—63
Ave.	449	146	361	—58
Basal ave.	278	141	217	—80

$$\% \text{ utilization } \frac{80-58}{449-278} \times 100 = 12.9$$

	Subject Jo		Weight in kg 64	
	Age 42	Sex F	Height in cm. 161	
Carrot 1	343	180	261	—98
2	407	173	282	—48
3	422	182	262	—22
4	437	220	216	+ 1
5	469	201	358	—90
6	421	190	309	—78
7	411	207	279	—75
Ave.	416	193	281	—58
Basal ave.	231	193	158	—120

$$\% \text{ utilization } \frac{120-58}{416-231} \times 100 = 33.5$$

	Subject Rd		Weight in kg. 74	
	Age 24	Sex M	Height in cm. 181	
Carrot 1	392	125	409	—142
2	460	129	431	—100
3	468	139	462	—133
4	491	148	463	—120
5	512	172	368	— 28
6	474	149	448	—123
7	461	175	484	—198
Ave.	465	148	438	—121
Basal ave.	289	142	288	—141

$$\% \text{ utilization } \frac{141-121}{465-289} \times 100 = 11.4$$

INTAKE	OUTPUT		BALANCE
	Urinary	Fecal	
Subject Bm		Weight in kg. 55	
Age 22	Sex F	Height in cm. 167	
mg.	*mg.*	*mg.*	*mg.*
383	276	238	—131
440	311	188	— 59
456	295	233	— 72
465	269	231	— 35
494	299	276	— 81
436	276	243	— 83
434	250	229	— 45
444	282	234	— 72
264	227	125	— 88

$$\% \text{ utilization } \frac{88-72}{444-264} \times 100 = 8.9$$

Subject Mh		Weight in kg. 61	
Age 24	Sex M	Height in cm. 166	
402	135	367	—100
436	134	511	—209
474	149	349	— 24
483	155	370	— 42
504	155	424	— 75
468	132	339	— 3
459	137	389	— 67
461	142	393	— 74
274	122	219	— 67

$$\% \text{ utilization } \frac{67-74}{461-274} \times 100 = 0.0$$

Subject Ws		Weight in kg. 65	
Age 22	Sex M	Height in cm. 171	
401	90	330	— 19
455	103	311	+ 41
473	111	512	—150
500	136	457	— 93
533	136	435	— 38
483	134	365	— 16
464	124	409	— 69
473	119	403	— 49
309	127	247	— 64

$$\% \text{ utilization } \frac{64-49}{473-309} \times 100 = 9.2$$

order. When these values are compared, according to the formula for computing percentage utilization (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41), with the individual intakes and the balances occurring in the carrot period, the following values for per cent utilization of carrot calcium are obtained: 17.6, 8.9, 12.9, 0.0, 33.5, 9.2 and 11.4. The mean of the values for the seven subjects is 13.4%.

DISCUSSION

The values reported here for the utilization of carrot calcium range from 0 to 34%. This range is great, and no tenable explanation presents itself for the fact that subject Mh made no use whatsoever of the carrot calcium whereas subject Jo utilized fully one-third of it. (Neither age nor sex difference could explain the variation.) On the other hand, the utilization figures for the five other subjects do not deviate greatly from the average; they range from 9 to 18%. In view of the fact that the values for the seven subjects do range from 0 to 34%, one well might question the worth of that 13.4% average in predicting the extent to which any one individual can utilize the calcium of carrots.

A comparison of these data and those obtained for these same subjects in a previous study on the availability of the calcium of milk (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41) indicates that the calcium of carrots was, as an average, only about one-half (55%) as available as was the calcium of milk. But the individual data are not consistent: only two (Ws and Rd) of the subjects approximated the average; three fell much lower; and two (Jo and Jd) showed virtually equal utilization with both sources. The values obtained for the utilization of the calcium of carrots and that of milk were, respectively, 18 and 15% for Jd, 9 and 31% for Bm, 13 and 35% for Hb, 0 and 18% for Mh, 34 and 30% for Jo, 9 and 20% for Ws and 11 and 20% for Rd. If one were to draw a general conclusion from this comparison, one would be inclined to state that adults probably do not utilize the calcium of carrots any more (and many undoubtedly even less) efficiently than they do that of milk.

In searching for the cause of the apparent inferiority in the availability of the carrot calcium, as demonstrated by the five subjects of the present study, one finds that the calcium of the two foods was not fed under strictly comparable conditions—i.e., the carrots were rich in "indigestible" carbohydrate,[4] whereas the milk was free of this substance. If fiber is capable of inhibiting calcium utilization, it could have been responsible for the low values observed in this study inasmuch as such large quantities were eaten. However, the effect could not have been brought about by a hastening of the passage of the fecal mass through the intestinal tract, since Jd was the only one of the seven subjects showing a decrease in the time required for the appearance of carmine, and she utilized carrot calcium better than she did milk calcium. Moreover, the literature reveals neither conclusive nor consistent evidence that the presence of fiber in the diet affects adversely the utilization of calcium.

None of the previously published calcium metabolism data obtained on human subjects ingesting carrots can be computed in terms of percentage utilization. There are studies, however, in which a comparison of the availability of the calcium of this food is made with that of milk. The studies of Sherman and Hawley ('22) and of Edelstein ('32), both of which used children as subjects, indicate poorer calcium retention when carrots were fed than when milk was the chief source of calcium. However, the data of Sherman and Hawley do not lend themselves to accurate evaluation with respect to the availability of the calcium of carrots since a mixture of spinach, celery and carrots was fed, and the poor calcium retention observed may have been due to the unavailability of the spinach calcium. Although one of Edelstein's infant subjects retained more calcium when fed carrots than he did when a "milchgriessbrei" constituted the main source of calcium, another child did better on the milk diet. The results of Rose

[4] Dr. T. S. Hamilton of the Animal Husbandry Department generously made analyses on the carrots and reported the following values based on wet weight: crude fibre, 0.99%; cellulose, 1.36%; and lignin, 1.59%.

('20) show the same lack of consistency—i.e., one of the women apparently made as good use of carrot calcium as she did of milk calcium, whereas the other woman retained more calcium when milk was fed. Obviously, none of this work on human beings gives substantial evidence concerning the comparative value of these two foods as source of calcium.

There are, however, two animal studies in which the observed data are overwhelmingly in favor of milk. One of these is the McClugage and Mendel ('18) study on two dogs; Shields, Fairbanks, Berryman and Mitchell ('40) on recalculating those data found that dogs A and B, respectively, utilized 52 and 40% of the calcium of milk but only 24 and 29% of the calcium of carrots—utilization only 60% as efficient as that found for milk calcium. The other study was carried out by the latter group of authors on twenty-two pairs of rats. Everyone of the rats receiving carrots retained less calcium than did its pair-mate which received an equivalent amount of calcium as milk; the mean value indicating only 85% as good usage. These data on animals not only indicate more efficient utilization of milk calcium over carrot calcium but also show greater consistency than those observed on man.

The carrot has ranked high as a source of dietary calcium ever since Rose ('20) reported that the calcium needs of man could be met "largely, if not wholly, from carrots." The facts demonstrated by the present study indicate that, for the subjects used here, the reverse is true. All of the subjects were in negative balance to a marked degree even though they were consuming as much as 700 gm. of carrots daily. On the basis of their individual abilities to utilize carrot calcium, these subjects would have had to consume an average of over 2 kg. of carrots daily in order to be in calcium equilibrium. Under practical dietary conditions, a 100 gm. portion would be considered an average-sized serving, and 200 gm. a generous one, but the latter quantity would add only 60 mg. of calcium to the intake, and, from this, the subject who had the highest rate of utilization could have secured only 20 mg.—a small fraction of the requirement. Why a discrepancy should exist

between the data of Rose and those herein reported is not clear. Obviously, the carrots used in Rose's study were richer in calcium than were the California-grown variety used in this study,[5] but this difference cannot be entirely responsible for the difference in the extent to which carrots met the requirement for calcium in the two experiments. A more likely explanation might be found in the state of calcium nutrition of the two groups of subjects. The fact that three of Rose's subjects, when ingesting no more than 383 mg. calcium daily, not only attained calcium equilibrium but even stored calcium would suggest that their tissues were not well saturated with calcium and were, perhaps, avid for it since the quantity of urinary calcium they excreted was very small. If Rottensten's study on rats ('38) is applicable to man and if Rose's subjects really were poorly nourished with respect to calcium, then one would suspect them of utilizing their calcium very efficiently. Such superior utilization would have made possible the satisfaction of their requirements with much smaller quantities of carrots than would have been needed by the seven subjects of the present study or by the nine men (assuming for them utilization rates for carrot calcium no greater than those found for milk) studied by Steggerda and Mitchell ('41). Whether or not many individuals exist who can do as well as did Rose's subjects is not known. One thing is clear however; if the adults of this study were to satisfy their calcium needs through the ingestion of this vegetable, they would have to eat such appalling quantities of it that they would have little digestive capacity for those other foods which are considered essential for health. It seems justifiable, therefore, to brand the carrot an unimportant source of calcium for these people.

SUMMARY

The extent to which the calcium of carrots could be utilized by adults was determined for four women and three men. Following the procedure previously used in this laboratory,

[5] See footnote 3, page 2.

two levels of calcium were fed, one averaging 270 mg. daily, the other 449. The same basal dietary was fed throughout the experiment, the higher level having been brought about by the daily ingestion of 700 gm. carrots, the total calcium content of which averaged 202 mg. The basal period lasted for 34 days and the carrot period for 39 days. On the basis of differences between the intakes of these two periods and differences in the magnitude of the negative balances, the amount of carrot calcium utilized was computed; the values obtained were 17.6, 8.9, 12.9, 0.0, 33.5, 9.2 and 11.4%, averaging 13.4. Five of these seven subjects showed poorer utilization of the carrot calcium than they did of milk calcium in a previous study.

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THE OCCURRENCE OF FREE AND BOUND BIOTIN[1]

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ONE FIGURE

(Received for publication August 14, 1941)

Biotin has recently assumed a position of great interest and importance in animal nutrition. du Vigneaud, Melville, György and Rose ('40) and György, Rose, Hofmann, Melville and du Vigneaud ('40) reported that it is identical with vitamin H, the factor curative of egg white injury in rats and chicks. It has been demonstrated by Williams and co-workers (Eakin, McKinley and Williams, '40; Eakin, Snell and Williams, '40; and György, Rose, Eakin, Snell and Williams, '41) that the biotin of the diet is fixed by an albumin fraction of raw egg white, "avidin", thus preventing the absorption of biotin from the intestinal tract and causing an induced deficiency. Biotin will also combine with "avidin" in vitro (Eakin, Snell and Williams, '40).

Hegsted, Oleson, Mills, Elvehjem and Hart ('40) observed in chicks a scaly dermatitis which was not due to pantothenic acid deficiency but was cured by concentrates of vitamin H. Hegsted, Mills, Briggs, Elvehjem and Hart ('41) have since found crystalline biotin to be completely curative. György

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation. Assistance was also received from the University of Wisconsin Works Progress Administration Natural Science Project.

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and Poling ('40) state that biotin is connected with the prevention of graying in rats and mice and Nielsen and Elvehjem ('41) have shown that biotin is involved in the spectacle eye syndrome of the rat.

McHenry and Gavin ('41) showed that beef liver fractions fed to rats caused acute fatty livers, the formation of which could not be prevented by administration of choline. Several biotin concentrates had a similar effect, hence biotin was indicated as the active factor. This effect demonstrates an important role for biotin in the synthesis of lipids by the rat.

In view of the growing significance of biotin in animal nutrition, additional information concerning its distribution and the forms in which it occurs in various materials would be highly desirable.

Snell, Eakin and Williams ('40) showed that biotin occurs, at least in liver, in a bound insoluble form, from which it could be freed by autolysis. We have already reported that acid hydrolysis of liver and kidney caused large increases in the amount of extractable biotin. In this paper we report the occurrence of bound biotin in a number of other tissues and its liberation by various means and present values for the biotin content of these tissues.

EXPERIMENTAL RESULTS

Assay method

Biotin was determined by a microbiological method (Peterson, McDaniel and McCoy, '40) employing Cl. butylicum no. 21 (Wisconsin). This organism on a basal medium of 2% glucose, 0.1% asparagine and 0.11% salts (K_2HPO_4, 10 gm.; KH_2PO_4, 10 gm.; $MgSO_4 \cdot 7\,H_2O$, 4 gm.; NaCl, 0.2 gm.; $MnSO_4 \cdot 4\,H_2O$, 0.2 gm.; $FeSO_4 \cdot 7\,H_2O$, 0.2 gm.) requires only the addition of biotin for maximum growth (Snell and Williams, '39). The measure of growth is the turbidity as read in an Evelyn photoelectric colorimeter. The organism requires anaerobic conditions for growth.

The stock culture is carried on sterile soil and a loopful is inoculated into a tube of the basal medium plus 0.25%

peptone. After 18–20 hours incubation, 10 cc. of this culture is centrifuged and resuspended in 10 cc. of sterile, freshly-boiled water. Three drops of this washed culture are added to each tube.

Lipless test tubes (25 × 200 mm.) are graduated at 36 cc. for the determination. A small amount of reduced iron is placed in each tube to aid in maintaining anaerobic conditions. The biotin solution for the standard curve or an extract of the material to be assayed, dissolved in 10 cc. or less, is placed in the tube and the volume brought to 11 cc. with water.

The basal medium is prepared in such concentration that addition of 25 cc. of it to each tube, i.e., to the 36 cc. mark, will give the desired concentrations. The pH of this basal medium should be 6.6–6.7 before addition. The tubes are plugged and autoclaved 30 minutes at 15 pounds pressure. They are cooled quickly and inoculated immediately. An oat-jar is prepared by filling a suitable container one-fourth full with oats, and adding just enough water to cover the oats. The assay tubes are placed in the jar and a glass top sealed on with plasticine. The respiration of the oats removes the oxygen and establishes a partial pressure of carbon dioxide. The jar is then placed in the incubator at 37°C. for 3 days.

After incubation the tubes are removed, shaken well and their turbidity read against the uninoculated basal medium set at 100. With excess biotin a reading of 15–20 is obtained. One-half of the difference between the inoculated control and the maximum reading, arbitrarily fixed as 20, is taken as half-maximum growth. A unit is defined as the quantity of material per cubic centimeter of medium which is required to give half-maximum growth. This unit is 0.000,010 micrograms (0.01 millimicrograms, or 0.01 mγ) for pure biotin, and by comparison of the unit weights, the biotin content of crude materials may readily be calculated. For example, if a tube containing the extract of 20 μg. of yeast per cubic centimeter gave half-maximum growth, the yeast contained 500 mγ of biotin per gram of dry substance.

As far as we have been able to determine, the organism is able to use any water-soluble biotin; that is, hydrolysis of such complex materials as molasses, peptone, and a liver autolysate caused no increase in the available biotin. It has become evident that there is considerable variation between duplicates even on pure biotin. The accuracy of the method is $\pm 20\%$.

A number of problems have been encountered in the use of the assay. If the pH of the basal medium before addition is brought to 6.3, a flocculent precipitate appears during autoclaving and incubation. This difficulty can be avoided by holding closely to a pH of 6.6–6.7. Care must be taken to prevent contamination of the culture. Whenever it seems to have occurred, growth is always maximum, i.e., the contaminants appear to have synthesized enough biotin to meet the requirements of the assay organism, although as judged by microscopic examination, the cells in such tubes consist mostly of the usual rods of *Cl. butylicum*. In an effort to avoid the cumbersome oat-jars and reduced iron, sodium thioglycollate was tested for maintenance of proper anaerobic conditions. The optimum concentration was 0.000,1 M, but even with this, many tubes failed to initiate growth. The addition of agar, cystine, or sodium hydrosulfite was also unsatisfactory.

Liberation of biotin

Detailed studies were made of the conditions required for the freeing of biotin from yeast and liver. Figure 1 shows one experiment with these two materials. The acid hydrolysis was done by autoclaving with 4N H_2SO_4. With liver the amount of water-soluble biotin reached a maximum at 1 hour and up to 4 hours no destruction was noted. A series was also run with 7N H_2SO_4. Here too, 1 hour was sufficient for complete liberation, and 4 hours treatment caused no destruction. With yeast 1 hour with 4N H_2SO_4 gave a maximum value but after 2–3 hours some destruction began.

Autolysis liberated biotin much more readily from yeast than from liver. Twenty-four hours autolysis, either with

toluene alone or with toluene and chloroform, freed 80–90% of the biotin of the yeast. The maximum was reached in 6 days and no destruction occurred during 3 weeks. No inhibition by chloroform of the freeing of biotin during autolysis as experienced by György ('39) was observed.

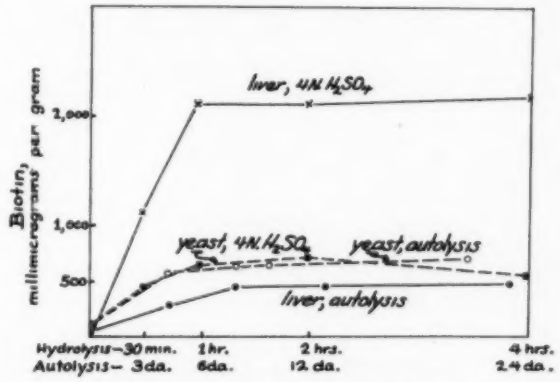

Fig. 1 Freeing of biotin in liver and yeast.

The release of the biotin from liver by autolysis was very slow and incomplete. Never more than 30% was rendered soluble by this method. Chloroform had no effect on the rate of liberation. The autolysis curve in figure 1 was run both with toluene alone and with chloroform and toluene and the values were identical.

The biotin content of samples of liver [2] is given in table 1. Autolysis again failed to free all of the biotin. Treatment with pepsin for 7 days at 50°C. also gave incomplete liberation. Other lengths of time and concentrations of pepsin were tested, but all were less effective than the example cited. Hydrolysis with trypsin for 3 hours at 50°C. completely liberated the biotin. Boiling of the liver to destroy the enzymes cut down the yield of biotin as did the use of a lower temperature. A hydrolysis with acid was run in our laboratories as a check and gave a figure in agreement with the maximum.

[2] Treated in various ways by the Difco Laboratories, Inc.

TABLE 1

Liberation of biotin from liver by various treatments.

TREATMENT	CONDITIONS	BIOTIN
		mγ/gm.
Autoclaved with water	1 hour, 15 pounds	60
Autolysis, in presence of chloroform and toluene	7 days, 37°C., pH 6.0–6.5	550
Pepsin	7 days, 50°C., pH 3.0	460
Trypsin	50°C., 3 hours	2,200
"	50°C., 3 hours (liver boiled first)	1,700
"	37°C., 7 days	1,800
"	37°C., 7 days, fresh trypsin added daily	2,100
H_2SO_4	4N, 1 hour, 15 pounds	2,140

Assays of biological materials

In table 2 are assays of the biotin content of a variety of natural materials illustrating the occurrence of free and bound biotin. The water extracts were made by suspending 1 gm. of the substance, homogenized by the method of Potter and Elvehjem ('36), in 50 cc. of water and autoclaving for 30 minutes. The suspension was adjusted to 50 cc., filtered, and the filtrate was properly diluted for assay. The acid extractions were carried out by autoclaving 1 gm. of the homogenized material with 50 cc. of 2N H_2SO_4 for 2 hours, and subsequently removing the $SO_4^=$ with $Ba(OH)_2$. From the work on liver and yeast, extraction with 4N H_2SO_4 for 1 hour seemed to be satisfactory, but because of occasional destruction of biotin with the 4N acid, 2N H_2SO_4 was subsequently adopted. Although maximal values were obtained under the conditions outlined, other conditions of extraction might give higher values.

Large increases, up to sixtyfold, were observed on hydrolysis of meats. Yeast, liver, kidney, egg yolk, and pancreas are all high in biotin, but lean meats are relatively low in the factor. Cow manure is rich in biotin but these samples do not approach the value of 150,000 mγ per gram given by Robbins and Schmidt ('39). Urine was assayed by addition

of the whole material to the medium. Preliminary hydrolysis of the urine did not increase the values given. In confirmation of the report of Snell, Eakin and Williams ('40), the biotin content of egg yolk is not increased by hydrolysis.

TABLE 2

Assays of natural materials.

MATERIAL	BIOTIN CONTENT, Mγ/GM. DRY MATTER	
	Water extract	Acid extract
Yeast and animal products		
Baker's yeast	27	700 (4N)
Brewer's yeast	100	830 ('')
Beef kidney	120	2500 ('')
Beef, lean	20	130 ('')
Cheese	26	24 ('')
Cow manure (winter ration)	500	490 ('')
Cow manure (pasture)	1700	1250 ('')
Egg white	530	480 ('')
Egg yolk (defatted)	1000	870 ('')
Heart	—	190 ('')
Milk	30–40 mγ/cc.	30–40 mγ/cc. (1N)
Meat scrap meal	12	48 (4N)
Pork kidney	100	4000 ('')
Pork liver	33	2000 ('')
Pork, lean	35	60 ('')
Pancreas	670	1230 (2N)
Urine (cow)	17 mγ/cc.	—
'' (human)	3.0 mγ/cc.	—
Vegetables and other green plant materials		
Alfalfa	260	280 (4N)
Beets	35	27 (2N)
Beans, string	200	230 ('')
Cabbage	62	32 (4N)
Carrots	400	285 (2N)
''Cerophyll'' (2 samples)	290–450	330–440 (4N)
Peas, fresh	95	110 (2N)
Potato	17	17 ('')
Spinach	450	480 (4N)
Tomato	1000	400 (2N)
Fruits		
Apple	43	60 (2N)
Banana	182	105 ('')
Orange	100	80 ('')
Peach	440	400 ('')
Raspberry	590	460 ('')
Seeds and products		
Barley	180	200 (4N)
Beans, navy	30	64 ('')
Corn (sample #1)	77	80 ('')
'' (sample #2)	50	50 ('')
'' (sample #3)	360	230 ('')
Corn steep	700	—
Oats (sample #1)	83	240 ('')
'' (sample #2)	71	170 ('')
Rice polish	170	270 (2N)
Rye	23	57 (4N)
Soybeans (sample #1)	200	540 (4N)
'' (sample #2)	83	830 ('')
'' (sample #3)	100	400 ('')
Wheat (sample #1)	22	67 ('')
'' (sample #2)	113	70 ('')
Wheat bran	83	140 (2N)
White flour	—	5.2 (2N)
Rice bran concentrate (Vitab)	1300 2000	— —
Nuts		
Peanut (roasted)	110	400 (4N)
'' (raw)	170	210 (2N) 200 (4N)
'' (raw)	110	210 (4N)
Pecan	80	114 (2N) 270 (4N)
Walnut, English	140	370 (2N) 280 (4N)

Assay of milk samples required special study. Addition of whole milk is impossible because of the precipitation of casein during fermentation. Repeated precipitation and washing of the casein gave a filtrate containing 98% of the biotin, but this was too tedious for a routine method. Hydrolysis of the milk for 1 hour with 1N H_2SO_4 gave a maximal value. A biotin recovery was run by adding 0.128 μg. of biotin to the milk before hydrolysis. Recovery was 104%. The simplest method, and the one finally chosen, was to dilute 5 cc. of milk to 50 cc. with an acetate buffer of pH 4.6. The casein was filtered off through a dry filter paper. This filtrate gave values equal to those by acid hydrolysis. The range of values was 30–40 μg./cc.

The biotin of green plants and vegetables exists in a free, water-soluble form. Some destruction by acid was noted with tomato. Alfalfa, string beans, carrots, spinach, and grass powders[3] are fair sources, while beets, cabbage, peas, and potatoes are low. The biotin of fruits is also in a water-extractable form. Peaches and raspberries rate high in this group, in contrast to apples and oranges which are low.

The picture in regard to seed products is extremely confused. Soybeans, navy beans, oats and rye appear to contain combined biotin, but barley and corn gave no increase on hydrolysis. The values for wheat are contradictory. Preliminary extractions with ether or chloroform, in order to permit better subsequent penetration by the acid, resulted in no improvement. The best method of preparation of grain samples for assay is still uncertain. A cereal concentrate[4] proved to be an excellent source of biotin.

The occurrence of firmly bound, water-insoluble biotin in nuts seems definitely established. Preliminary ether or chloroform extractions again yielded no improvement. Values of 200–400 mγ per gram were obtained for all of the samples.

[3] Cerophyll.

[4] Vitab. Rice Bran Concentrate.

Destruction of biotin by acid

During treatment of crude materials with 4N H_2SO_4, destruction of biotin has been frequently observed. None has been found with 1N acid and only a few cases with 2N. Evidence of partial destruction was mentioned in the discussion of yeast and low values with acid were noted for cow manure, tomatoes, bananas and nuts. Table 3 shows experiments re-

TABLE 3

Acid destruction of biotin.

SAMPLE	BIOTIN (mγ/GM. DRY MATTER) Time of acid treatment				
	None	30 min.	1 hour	2 hours	4 hours
Molasses concentrate, 4N H_2SO_4	2640	2200	—	820	720
Corn + 1460 mγ of added biotin, 4N H_2SO_4	1540	1540	—	1000	840
Dried juice of young barley					
1N H_2SO_4	570	480	500		
2N H_2SO_4		370	280		
4N H_2SO_4			25		

garding the destruction of biotin in three different materials. Destruction with acid was not confined to any single type of material, some of the samples being high in protein, some in carbohydrate, and others in lipids. However, it was evident that a considerably longer time was required to produce any destruction in yeast and animal tissues.

SUMMARY

The biotin of yeast and animal products occurs primarily in a water-insoluble firmly-bound combination. Increases of from two- to sixtyfold occurred on hydrolysis. Vegetables, green plant materials, and fruits contain a water-extractable form, while seeds and nuts appear to have considerable amounts of bound biotin.

The biotin may be freed either by hydrolysis with acid, 2N H_2SO_4 for 2 hours being the generally optimal treatment,

or with enzymes such as trypsin. Autolysis is excellent for liberating the biotin from yeast.

Under treatment with 4N H_2SO_4, destruction of biotin has been found in yeast, corn, barley-juice powder, and soybeans.

ACKNOWLEDGMENTS

Much of the early work on the development of the method of water extraction of biotin from biological materials was done by L. E. McDaniel. We are also indebted to A. A. Kline for assistance in some of the analyses. We wish to thank Dr. H. G. Dunham of the Difco Laboratories Inc. for his cordial cooperation in the preparation of generous quantities of the liver samples. The samples of crystalline biotin used as standards were generously furnished by Professors F. Kögl and V. du Vigneaud.

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STUDIES IN NICOTINIC ACID METABOLISM

I. THE FATE OF NICOTINIC ACID IN MAN [1]

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A large number of publications have appeared within the last 3 years (reviewed by Lepkovsky, '40, and Morgan, '41) presenting quantitative data on the excretion of "nicotinic acid" in the urine of man and animals under a variety of conditions. Obviously, the figures and their interpretation depend almost entirely upon the specificity of the given methods and upon the relative yield of color reacting substances from techniques employed in the splitting or the transformation of the various nicotinic acid derivatives into nicotinic acid itself.

In considering the question of specificity, pyridine and its derivatives, including nicotine in the urine of smokers, demand attention. In the majority of reports in which the original König color reaction with CNBr and aniline was used, the relatively high daily outputs of 10 to 30 mg. of "nicotinic acid" in human urine could be traced to the presence of variable amounts of nicotine and of other pyridine derivatives in the urine.

[1] The data in this article are taken from a thesis to be presented by Herbert P. Sarett in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences of Duke University.

A preliminary report was made by the authors before the meeting of the American Society of Biological Chemists at Chicago, April, 1941.

[2] Upjohn Fellow in Biochemistry.

Of the known nicotinic acid derivatives, only nicotinuric acid and trigonelline have been well established as constituents of human and dog urine (Ackermann, '12; Linneweh and Reinwein, '32 a and '32 b; Perlzweig, Levy and Sarett, '40; Melnick, Robinson and Field, '40). Euler and Schlenk ('39) and H. Kohn and P. Handler (personal communication) have found the concentration of coenzyme in urine to be below the sensitivities of their methods. Calculated from Kohn's data, not more than 4% of the acid hydrolyzable nicotinic acid derivatives could be in the form of coenzyme. Our own studies indicate that free nicotinic acid and nicotinamide could account for only 25 to 40% of the acid hydrolyzable nicotinic acid derivatives in normal urine, and even a smaller proportion after the administration of relatively large doses of nicotinic acid or amide (see below). The two derivatives which predominate in human and in dog urine, both normally and after the administration of either nicotinic acid or the amide, are nicotinuric acid and trigonelline. Trigonelline, which constitutes over 90% of the measurable nicotinic acid derivatives in normal urine (the remainder comprising the acid hydrolyzable fraction) also accounts for 60 to 95% of these derivatives after the administration of either nicotinic acid or the amide. Although the acid hydrolyzable nicotinic acid derivatives make up a small fraction of the total excretion in normal urine, over one-half of this fraction, as judged from the data in table 2, appeared to be in the form of nicotinuric acid. This is contrary to the statement of Melnick, Robinson and Field ('40) that no nicotinuric acid is found in normal human urine.

Recognizing these facts, all techniques and methods employed for the determination of nicotinic acid and its derivatives in urine must be so designed and the conditions so defined as to ensure the quantitative measurement of all of these nicotinic acid derivatives and not of any extraneous pyridine compounds. Since with these color reactions nicotinamide and nicotinuric acid yield variable intensities of the same color as obtained with nicotinic acid, they must first be completely

hydrolyzed to nicotinic acid by boiling with strong acid. Trigonelline can only be measured quantitatively after a strong alkali-urea hydrolysis following the acid hydrolysis.

Furthermore, since trigonelline is a normal constituent of a number of plant tissues and foods, notably coffee and the leguminous seeds (Maier-Bode and Altpeter, '34), the intake of these in the diet must either be excluded or controlled in metabolism experiments.

In the investigations reported in this and in the subsequent communication (Sarett, '42) we have been guided by the above considerations, while being fully aware of the fact that the measurement of the nicotinic acid derivatives mentioned above may not yet yield a complete picture of the fate of nicotinic acid in the human and animal organisms, as will be apparent from the presented data.

This communication deals with a study of the fate of nicotinic acid in normal man under controlled dietary conditions, before and after the administration of various nicotinic acid derivatives.

EXPERIMENTAL

The experiments reported here are divided into three parts. One series consists of the experiments performed on three normal individuals (the authors) who were maintained on a rigorously controlled diet consisting of trigonelline free foodstuffs. Levels of daily excretion of nicotinic acid and trigonelline on this diet and after oral doses of nicotinic acid derivatives were determined (table 1).

The second series of experiments was a set of analyses of the urines of thirty-three medical students, who were on various diets low in trigonelline, and of three fasting students (table 3).

The third series was a set of saturation tests with nicotinamide on six normal young adults (table 4).

All urines analyzed were 24-hour specimens which were collected under toluene and kept in the refrigerator. The acid hydrolyzable nicotinic acid fraction (NA) and trigonelline

were determined by the previously described method (Perlzweig, Levy and Sarett, '40). Since publication it has undergone the following minor modification and improvement:

After boiling a suitable urine sample [3] with 10 cc. of 7 *N* HCl and 0.25 cc. of concentrated HNO_3 over a microburner for 1 hour, there is very little charring and the pigments are largely decolorized. The nitric acid, however, produces a light yellow color in urine which is not removed by the subsequent procedures. This difficulty is overcome by extracting the acid digests, in 25 ml. glass stoppered graduated cylinders, with two or three 5 cc. portions of ether. The ether portions, containing most of the yellow pigments, are pipetted off; the ether remaining in solution in the digests is removed by immersing the cylinders into a beaker of warm (40–50°C.) water; after cooling, the solutions are adjusted to a convenient volume in the cylinders. This extraction with ether results in excellently low blank values in the final stage of the colorimetric determination. The ether extraction was shown not to cause any losses of nicotinic acid or of trigonelline. The rest of the analytical procedure as previously described remains unaltered.[4]

NA and trigonelline excretion after administration of nicotinic acid and derivatives

On a low trigonelline diet consisting of meats, white bread, rice, corn, milk, milk products and fruits, three subjects excreted 1.1 to 1.5 mg. of NA per day and 9 to 13 mg. of trigonelline per day (table 1).

[3] In the presence of relatively large amounts of nicotinuric acid, correspondingly smaller aliquots of urine were taken and the boiling continued for 90 minutes. If the nicotinuric acid is not completely hydrolyzed to nicotinic acid by the acid digestion, it will be split in the alkali urea hydrolysis of trigonelline, and will be erroneously measured as trigonelline.

[4] The most uniformly reproducible results in the final color reaction were obtained by using a 3% solution of crystalline CNBr (Eastman). This solution keeps well in dark amber glass in the refrigerator for at least 3 weeks and possibly longer.

TABLE 1

Excretion of NA and trigonelline by three subjects, on a "trigonelline free" diet, after ingested doses of nicotinic acid compounds.

	DAYS	SUBJECT H NON-SMOKER			SUBJECT S MODERATE SMOKER			SUBJECT P HEAVY SMOKER		
		NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Control	1	1.4	12.2	12.4	1.1	13.0	12.8	1.4	12.0	12.2
	2	1.2	9.0	9.3	1.5	12.2	12.7	1.4	11.0	11.3
After 200 mg. dose of nicotinic acid	3	13.5	27.9	38.6	6.9	39.3	42.3	21.0	38.3	55.5
	4	1.0	19.1	18.2	1.2	18.2	17.6			
After 200 mg. dose of nicotinamide	9	1.3	51.0	47.3	1.6	68.0	62.6	2.0	45.0	42.5
	10	1.4	22.7	21.8	1.1	24.5	23.1	1.5	17.8	17.5
After 200 mg. dose of trigonelline	11	1.2	181.0		1.2	170.0		1.9	196.0	
	12	1.0	18.5		1.1	19.4		1.4	26.0	

[1] NA includes all nicotinic acid derivatives hydrolyzable by boiling with 7 N HCl.

[2] Total represents the total excretion of nicotinic acid and trigonelline, calculated in terms of nicotinic acid.

These data were substantiated by the values of 8 to 12.6 mg. of trigonelline excreted per day by three individuals after 3 days of starvation (table 3) and show that the dietary trigonelline was reduced to a minimum. The figures for NA given here and in the other tables represent $HCl–HNO_3$ acid hydrolyzable derivatives of nicotinic acid as described above, and include the nicotinic acid, nicotinamide, coenzyme and nicotinuric acid which may be present in the urine.

The daily dietary nicotinic acid intake of the three subjects was kept at a fairly constant level of about 30 to 40 mg.[5] Test doses of nicotinic acid derivatives were equally divided between breakfast and lunch and were taken orally at the end of these meals.

Two hundred milligram doses of nicotinic acid, taken on the third day (table 1) resulted in the added excretion of 35

[5] An average daily diet included 300 gm. of meats containing about 60 to 70 μg. NA per gram, and 2 kg. of cereals, breads and vegetables, which contained roughly about 8 to 10 μg. NA per gram.

to 44 mg. of total NA and trigonelline in the next 2 days.[6] The average for the days of the control period served as a base line for these calculations. This added excretion represented only 17 to 22% of the ingested dose. Of the total extra excretion 55 to 84% was in the form of trigonelline. The remaining 16 to 45% was entirely nicotinuric acid. This was demonstrated in the following manner.

Nicotinuric acid was completely hydrolyzed by the 7 *N* HCl–HNO_3 mixture which was used for nicotinic acid determinations. After treatment of nicotinuric acid in a water bath for 1 hour with 1 *N* HCl, the Bandier color reagents (Bandier and Hald, '39) gave only 35% of the color of the equivalent amount of nicotinic acid. Nicotinamide, on the other hand, was completely hydrolyzed by 1 *N* HCl or the 7 *N* HCl–HNO_3 treatment. Two equations have been set up:

Nicotinuric acid + other nicotinic acid derivatives = 7 *N* HCl value

0.35(nicotinuric acid) + other nicotinic acid derivatives = 1 *N* HCl value

Upon subtraction there was obtained the equation:

0.65 (nicotinuric acid) = 7 *N* HCl value − 1 *N* HCl value

Typical figures for the partition of the NA fraction of normal urines and those obtained after doses of nicotinic acid are given in table 2.

TABLE 2

Partition of NA fraction in normal urines and after dose of nicotinic acid, by 1 N HCl and 7 N HCl hydrolyses. Explanation in text.

	NICOTINIC ACID			NICOTINURIC ACID
	After 1 *N* HCl	After 7 *N* HCl	As nicotinuric acid	
	μg./cc.	*μg./cc.*	*μg./cc.*	%
Normal urines	0.7	1.4	1.1	78
	0.9	1.5	0.9	60
	0.9	1.8	1.4	78
Urines after dose of nicotinic acid	13.0	41.0	43.0	105
	26.4	72.0	70.0	97
	7.2	19.2	18.4	96

[6] It is to be noted that while the NA fractions returned to a normal level on the second day after each dose of nicotinic acid or the amide, the trigonelline was more slowly excreted, maintaining a higher level in the urine for several days.

Since nicotinuric acid is the glycine conjugate of nicotinic acid, and since trigonelline is a methylated derivative of nicotinic acid, the effects of administrations of 2 gm. of glycine and of 400 mg. of choline in combination with 200 mg. nicotinic acid on the relative proportions of urinary trigonelline and nicotinuric acid were tried. No demonstrable effects of these additions were observed in the partition of the nicotinuric acid and trigonelline fractions in the urine in all three subjects. Likewise upon repeating these experiments in dogs (Sarett, '42) but using much larger amounts of glycine and choline no effect was observed.

Of the 200 mg. of nicotinamide taken on the ninth day, 18 to 30% could be accounted for by the added trigonelline excretion of the following 2 days. Distinctly different from the excretion after a dose of nicotinic acid was the lack of any increase in the NA fraction. To show that the question of absorption of nicotinic acid or nicotinamide from the gastrointestinal tract did not control these results, nicotinamide was given intravenously, with the same resultant excretion as that obtained after the oral dose.

Trigonelline ingested in 200 mg. doses was almost completely excreted (80–90%) in the following 2 days. Nicotinuric acid taken orally in 100 mg. doses by the three subjects was excreted, in unchanged form, to the extent of 56, 60 and 65%, with no concomitant increase in the trigonelline fraction. The utilization of nicotinuric acid by man thus appears to be doubtful, but because of its relative insolubility the complete absorption from the gastrointestinal tract is questionable. A more definite solution is being sought by means of parenteral administration of nicotinuric acid.[7]

Levels of nicotinic acid and trigonelline excretion of thirty-three medical students

In the course of their metabolic experiments on the effects of various dietary regimes upon the composition of the urine,

[7] Since this paper has gone to press it has been determined that nicotinuric acid given intravenously in 100 mg. doses to three subjects was excreted to the extent of 96, 94 and 97%.

thirty-three medical students who did not drink coffee were asked to abstain from leguminous foods. These regimes included normal, high protein, high carbohydrate-low protein diets, high water intake and starvation. The range and averages of NA and trigonelline excretions are shown in table 3.

TABLE 3

Daily excretion of NA and trigonelline by young normal adults on low trigonelline diet and in starvation.

	NA		TRIG.	
	Range	Average	Range	Average
	mg.	*mg.*	*mg.*	*mg.*
Various diets				
33 subjects (see text)	0.7–2.2	1.2	5–25.4	15.1
Starvation—Subject 1		1.1		9.3
Subject 2		2.0		12.6
Subject 3		1.4		8.2

The diets of these students could not be as rigorously controlled as were the diets of the first three subjects, and this resulted in several (seven) cases of high levels of trigonelline excretion. The average trigonelline excretion, omitting these questionable values was 15.1 mg. per day. The average for the more rigorously controlled diet (table 1) was 11 mg. trigonelline per day. The difference was due to the diet. The 8 to 12.6 mg. of trigonelline (average, 10 mg.) excreted by three individuals on the third day of fasting was an excellent check of the validity of the above values.

In this group of thirty-three students the NA excretion ranged from 0.7 to 2.2 mg. per day with an average daily excretion of 1.2 mg. Normal urines analyzed by our method have always given values in this range, in smokers as well as non-smokers. In the six habitual smokers in this group the NA values ranged from 1.0–2.2 mg., average 1.2; the trigonelline values ranged from 10.4 to 15.5, average 12.8 mg. These values do not differ in any significant manner from those obtained on the group as a whole.

The 24-hour urine of a 7-week-old premature baby weighing 3.1 kg., who was on a trigonelline free diet of milk and lactose, contained 0.14 mg. NA and 2.5 mg. of trigonelline.

On the basis of body weight the levels of NA and trigonelline excretion were much higher than those in the adult.

Saturation tests with nicotinamide

In all of the above experiments test doses of nicotinic acid and nicotinamide had been administered to normal individuals in a good nutritional state. Of the 200 mg. of nicotinic acid or amide ingested only 12 to 45% (average 22%) was accounted for by the added urinary NA and trigonelline. There were wide individual variations.

The ingested nicotinic acid which could not be recovered in the urine either went through another metabolic pathway or was taken up by the tissues which were not fully saturated. In dogs, which had been saturated with nicotinic acid, all of the extra administered nicotinic acid could be recovered in the urinary NA and trigonelline (Sarett, '42). In man this has not been possible to achieve thus far, as shown in the following experiment.

Six medical students were given 500 mg. of nicotinamide orally for 6 successive days. They abstained from trigonelline containing foods during this time. The urines of the first and sixth days were collected and analyzed. The results are summarized in table 4. The control urines were the normal urines collected in the preceding part of the experiment.

TABLE 4

Saturation tests on six individuals receiving 500 mg. nicotinamide per day for 6 days on low trigonelline diet.

SUBJECT NUMBER	CONTROL URINE			URINE OF FIRST DAY AFTER 500 MG. NICOTINAMIDE				URINE ON SIXTH DAY			
	NA [1]	Trig.	Total [1]	NA [1]	Trig.	Total [1]	Per cent recovery [2]	NA [1]	Trig.	Total [1]	Per cent recovery [2]
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	%	*mg.*	*mg.*	*mg.*	%
32 B	0.8	12.8	12.3	5.4	105	100	18	7.3	182	171	32
32 C	0.9	8.5	8.5	10.0	104	104	19	8.0	233	218	42
14	1.2	16.4	15.9	4.9	103	98	17	4.6	163	151	28
40	2.2	12.5	13.4	4.2	110	103	18	7.6	164	155	28
48	1.1	39.4	36.5	10.3	142	138	21	11.8	240	228	40
50	1.6	20.7	20.2	1.0	50	46	5	6.4	169	158	27

[1] See table 1.

[2] Per cent recovery refers to the portion of the 500 mg. dose of nicotinamide accounted for by the extra total excretion of NA and trigonelline.

In all cases, both on the first and sixth days, trigonelline comprised over 90% of the added total excretion. Of the remaining NA fraction only 65 to 80% was nicotinuric acid. This differs from the results obtained after the administration of nicotinic acid, in which the NA fraction was proportionately larger and was composed entirely of nicotinuric acid.

On the first day 17 to 21% of the ingested dose could be accounted for in the urines of five of the subjects, but only 5% in the urine of subject no. 50. On the sixth day, it was possible to account for 27 to 42% of the dose in this way.[8] Although this showed an increase in nicotinic acid saturation of the individuals, it also indicated that another pathway for nicotinic acid metabolism may exist in man.

The small output of NA and trigonelline by subject 50 after the first dose of 500 mg. of nicotinamide indicated the possibility of a deficiency in nicotinic acid. This was tested by giving 500 mg. of nicotinamide to ten more normal people all of whom responded with an excretion of NA and trigonelline of at least 17 to 20% of the test dose. In nutritionally deficient hospital patients, however, only a small portion (0 to 5%) of the nicotinamide could thus be accounted for, and in some of these patients several large doses of nicotinamide were necessary before any increase in NA and trigonelline could be detected.

Further results obtained on thirty unselected patients upon admission to the hospital indicate that it is possible to establish a test on this basis as a means for detection of nicotinic acid deficiency in man. This work is now in progress.

SUMMARY

Trigonelline and nicotinuric acid are the two known end products of nicotinic acid metabolism found in human urine.

For a study of normal trigonelline excretion in man, a diet free of coffee, peas, beans and other legumes is necessary. On

[8] After the daily intake of 500 mg. nicotinamide for 30 days, three normal subjects excreted no more than 40% of the ingested dose.

such a diet, rigorously controlled, three individuals excreted 9 to 13 mg. of trigonelline per day. On less rigorously controlled diets, twenty-six students excreted an average of 15 mg. of trigonelline per day. Three individuals, after 3 days of fasting, excreted 8 to 12.6 mg. trigonelline per day.

The normal daily excretion of acid hydrolyzable nicotinic acid derivatives (NA) by thirty-six subjects on different diets and under fasting conditions was 0.7 to 2.2 mg. This level was not affected by smoking.

There is a constant loss of at least 10 mg. of known nicotinic acid derivatives in the urine daily, regardless of diet.

Of 200 mg. oral doses of nicotinic acid, 25 to 90 mg. could be accounted for by the increase in urinary trigonelline and nicotinuric acid. Trigonelline accounted for 55 to 84% of this increase. The remainder was nicotinuric acid. The feeding of glycine and choline with 200 mg. doses of nicotinic acid did not change the proportions of trigonelline and nicotinuric acid excreted.

Of 200 mg. doses of nicotinamide, 36 to 60 mg. could be accounted for by the increase in urinary trigonelline. There was no increased excretion in the acid hydrolyzable nicotinic acid fractions.

Trigonelline is not utilized by man when taken orally, and is excreted almost completely as such. Nicotinuric acid when ingested is excreted to the extent of about 60%, unchanged, and without an accompanying increase in trigonelline.[9]

After six daily doses of 500 mg. of nicotinamide the urinary excretion of NA and trigonelline in six subjects accounted for 27 to 42% of the ingested dose, compared to 5 to 21% which could be accounted for after the first day. Over 90% of this added excretion was in the form of trigonelline. Of the remaining NA fraction, 65 to 80% was nicotinuric acid.

The extra excretion of NA and trigonelline after a test dose of nicotinamide may serve as an indication of the nutritional status of an individual with respect to nicotinic acid.

[9] See footnote 7, page 29.

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STUDIES IN NICOTINIC ACID METABOLISM

II. THE FATE OF NICOTINIC ACID IN NORMAL AND BLACK TONGUE DOGS [1]

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There are many similarities in man and dog with respect to the need for nicotinic acid and the fate of this substance. The avitaminotic diseases, pellagra and black tongue, which result from the deficiency of nicotinic acid have many like characteristics. Both man and dog can form nicotinuric acid and trigonelline from nicotinic acid (Ackermann, '12; Sarett, Huff and Perlzweig, '42; Melnick, Robinson and Field, '40).

This communication deals with a study of the nicotinic acid and trigonelline excretion of dogs both at different levels of nicotinic acid intake and in black tongue. Since urinary trigonelline may be attributed both to nicotinic acid metabolism and to the ingestion of many foods containing trigonelline, it was necessary to carry out these experiments with a trigonelline free diet (Sarett, Huff and Perlzweig, '42; Perlzweig, Levy and Sarett, '40).

The level of trigonelline excretion drops markedly after a few weeks on a black tongue producing diet, and practically disappears with the onset of black tongue. More striking

[1] The data in this article are taken from a thesis to be presented by Herbert P. Sarett in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences of Duke University.

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than the difference in level of trigonelline excretion, however, is the difference between the complete retention of large doses of nicotinic acid by the black tongue dog and the complete excretion of this compound by the saturated animal. Most of this excretion is in the form of trigonelline, as already reported for man (Sarett, Huff and Perlzweig, '42).

EXPERIMENTAL

Full grown dogs (10–14 kg.) were kept in individual metabolism cages and the urines were collected daily under toluene and analyzed for nicotinic acid and trigonelline by the previously described method (Sarett, Huff and Perlzweig, '42; Perlzweig, Levy and Sarett, '40). The only modification of the method was the extraction of the acid hydrolysate with small amounts of ether (5 cc.) to help insure low blanks. This was particularly advisable when the nicotinic acid and trigonelline content of the urine became so low that it was necessary to take 40 to 50 cc. of urine for analysis.

For the low nicotinic acid diet, the black tongue producing ration of Koehn and Elvehjem ('37) was used, since we were unable to detect trigonelline in either the casein or the corn meal. It is possible that very small amounts of trigonelline are contained in these foods, but this would not be enough to make a significant difference either in the figures or their interpretation. Crude casein was substituted for the purified casein, since the nicotinic acid content of the crude product was only about 2 μg. per gram. The yellow corn meal contained about 8 μg. nicotinic acid per gram. The percentage composition of the diet was as follows: yellow corn meal 72, casein (crude) 18, cottonseed oil 5, cod liver oil 2, calcium carbonate 1, calcium biphosphate 1, sodium chloride 1. This mixture was cooked with four parts of water, so that each kilogram of cooked food contained 200 gm. of this mixture. On this basis, the nicotinic acid content of the diet was 1.2 mg. per kilogram. The dogs received weighed amounts of the food corresponding to approximately 1 kg. cooked food per 10 kg. body weight or 0.1 to 0.2 mg. nicotinic acid per kilogram body weight. A supple-

ment of 2 mg. of riboflavin and 2 mg. of thiamine chloride was given each dog every week.

Excretion on a low nicotinic acid diet

Three healthy dogs were fasted for 4 days before receiving the low nicotinic acid diet. At the end of this time they excreted 0.3 to 0.7 mg. NA and 3.3 to 7.8 mg. trigonelline per day (table 1). The low nicotinic acid diet was fed for 20 days,

TABLE 1

Daily urinary excretion of NA and trigonelline of three dogs on a diet low in nicotinic acid.

DAY OF DIET	A — 14.8 KG.			B — 10.7 KG.			C — 11.2 KG.		
	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
After 4 days starvation	0.7	7.6	7.5	0.5	7.8	7.5	0.3	3.3	3.3
1–5	0.7	2.6	3.0	0.6	2.5	2.8	0.6	3.2	3.5
18–20	0.6	1.6	2.0	0.5	2.7	2.9	0.5	1.1	1.5
25 mg. nicotinic acid orally on twenty-first day									
21	1.2	3.1	4.0	1.5	8.1	8.8	0.8	1.6	2.2
22	1.1	2.8	3.6	0.7	3.5	3.8	0.4	0.8	1.1
25 mg. nicotinic acid intravenously on twenty-fourth day									
24	2.7	6.1	8.2	1.2	4.8	4.3	0.8	1.8	2.4
25	1.0	4.4	5.0	0.9	5.5	5.8	0.6	1.4	1.9
25 mg. nicotinamide intravenously on twenty-seventh day									
27	3.9	9.8	12.7	1.3	10.6	10.8	0.6	6.4	6.4
28	1.6	8.1	8.9	0.9	6.4	6.7	0.6	2.2	2.6

[1] NA includes all nicotinic acid derivatives hydrolyzable by boiling with 7 *N* HCl.
[2] Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

during which time the dogs continued to eat approximately the same amount of food per day and increased their weights almost back to the pre-fasting level. During these 20 days the NA excretion of the dogs changed very little, but the trigonelline dropped to 1.1 to 2.7 mg. per day.

An oral dose of 25 mg. of nicotinic acid at this time was almost completely retained by each of the three dogs. Of the

25 mg., 18.2 to 24.3 mg. were retained, as estimated by the following 2 days' excretion. The excretion during the eighteenth to twentieth days serves as a baseline for the calculations. A second 25 mg. dose of nicotinic acid was given intravenously 3 days later, and here the dogs retained 16.0 to 23.8 mg. Three days later a third dose of 25 mg. nicotinamide was substituted for the nicotinic acid and in this case only 7.4 to 19.2 mg. of the dose was retained. The greater part of the amount which was excreted was trigonelline. Most of the extra excretion in the NA portion was in the form of nicotinuric acid.

Although it was logical to expect a greater retention of nicotinic acid after the first oral dose, the question of efficient absorption could not be overlooked. To investigate this, the feces were analyzed and showed no differences in NA and trigonelline content, either before or after an oral dose of nicotinic acid was given.

The feces were collected at frequent intervals and kept in 5 N HCl. The specimens for the eighteenth to twentieth days inclusive were pooled to give a basal sample, and those passed during the 3-day period following the oral dose of 25 mg. nicotinic acid likewise pooled and examined for any increases. The feces were homogenized and analyzed for NA and trigonelline in a manner similar to that used for urine.

The fecal NA of the dogs on the basal diet was 0.6 to 1.6 mg. per day and the trigonelline only 0.15 to 0.3 mg. per day. After the 25 mg. oral dose the NA was 0.7 to 1.1 mg. per day and the trigonelline 0.1 to 0.3 mg. per day. Analyses of feces of these dogs on a high nicotinic acid diet (1.1 mg. per kilogram) gave similar results. Most likely this fecal NA and trigonelline was entirely of bacterial origin.

Excretion on high nicotinic acid diet

For the high nicotinic acid diet, 10 mg. of nicotinic acid were added to each kilogram of cooked food. This was checked by analysis of the cooked food. Thus each dog eating 1 kg.

of food per 10 kg. body weight was ingesting approximately 1.1 mg. nicotinic acid per kilogram per day.

The three dogs used in the low nicotinic acid experiment became the subjects in this experiment immediately after the third 25 mg. dose of nicotinamide. Another dog (D) which had been on the same low diet for other experiments was also included. After 9 days on this diet there was a rapid increase in trigonelline excretion and very little change in NA excretion (table 2). On the sixth to ninth days the dogs

TABLE 2

Daily urinary excretion of NA and trigonelline of four dogs on a diet high in nicotinic acid (1.1 mg. per kg. body weight).

DAY OF DIET	A — 14.5 KG.			B — 10 KG.			C — 11.1 KG.			D — 11.8 KG.		
	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]	NA [1]	Trig.	Total [2]
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1–3	1.1	5.2	5.8	0.4	1.1	1.4	0.5	2.7	2.9	0.4	1.5	1.7
4–6	1.4	5.1	6.0	0.5	2.6	2.8	0.5	4.9	4.9	0.4	4.3	4.3
7–9	1.6	7.3	6.2	0.5	4.9	4.9	0.6	5.0	5.1	0.5	4.1	4.2
10	25 mg. nicotinic acid intravenously											
10	5.7	29.3	32.1	1.6	22.7	22.0	2.8	19.8	20.6	1.0	23.6	22.2
11	1.9	15.7	16.0	0.4	8.7	8.2	0.6	11.8	11.2	0.4	11.3	10.6
12	1.2	7.1	7.6	0.6	7.0	6.9	0.5	6.9	6.7	0.4	6.8	6.5
13	100 mg. nicotinic acid intravenously											
13	17.1	43.2	56.0	17.8	84.5	93.8	19.7	70.0	82.7	5.3	53.8	53.7
14	7.5	63.0	64.1	1.0	26.9	25.2	1.1	21.7	20.6	1.3	58.0	53.5
15	1.8	16.2	16.4	0.5	8.9	8.5	0.5	10.9	10.3	0.4	10.5	9.9
16	1.4	13.1	13.2	0.4	5.5	5.4	0.5	9.3	8.8	0.4	7.2	6.9

[1] NA includes all nicotinic acid derivatives hydrolyzable by boiling with 7 *N* HCl.

[2] Total represents the sum of NA and trigonelline calculated in terms of nicotinic acid.

excreted a total of 4.2 to 8.2 mg. in the form of NA and trigonelline on an intake of 12 to 15 mg. per day.

At this time a 25 mg. intravenous dose of nicotinic acid was almost completely excreted; dog A apparently retained none and the others retained about 5 to 8 mg. It was impossible to calculate this retention accurately since the basal excretion varied somewhat. However, for the purpose of these calculations we arbitrarily took the twelfth day as the basal

level of excretion for the dogs on this diet. It is to be remembered that these dogs retained 5 to 10 mg. of the nicotinic acid received in the diet each day, and that the retention and excretion calculated in all of these figures were only for the extra doses of nicotinic acid or nicotinamide.

On this basis the added excretion of total NA and trigonelline (calculated as nicotinic acid) after the first 100 mg. intravenous dose of nicotinic acid was 112, 115, 93 and 96 mg. A second 100 mg. dose given 3 days later resulted in the same amount of added excretion. After receiving intravenous doses of 100 mg. of nicotinamide there were excreted 103, 108, and 105 mg. extra of total NA and trigonelline. These dogs had reached the saturation point. They no longer retained any part of a large dose of nicotinic acid or nicotinamide, but converted all of it into two products—trigonelline and nicotinuric acid. In our experiments on humans (Sarett, Huff and Perlzweig, '42) after repeated dosing with nicotinamide only 27 to 42% of the dose could be accounted for in the urine. There may be another pathway of nicotinic acid metabolism in the human which differs from that in the dogs.

In the urine of dogs trigonelline made up 75 to 94% of this extra excretion when either nicotinic acid or nicotinamide was given. When nicotinic acid was given the 6 to 25% which was excreted in the NA fraction was practically all in the form of nicotinuric acid. However, when nicotinamide was given, only 75 to 85% of this fraction was nicotinuric acid.

In an attempt to influence the proportions of trigonelline and nicotinuric acid formed, choline and glycine, in separate experiments, were added to the diet. Fifteen grams of glycine fed to each of two dogs (B and C) during 3 days preceding an intravenous dose of 100 mg. of nicotinic acid resulted in the same distribution of the NA and trigonelline fractions in the urine as that shown for these dogs in table 2 after a similar dose of nicotinic acid alone. Similarly 4 gm. of choline fed to each of these dogs during 2 days preceding another dose of nicotinic acid did not affect the partition of these fractions in

the urine. Thus the ingestion of large amounts of glycine and choline appears to have no appreciable effect upon the excretion of nicotinuric acid and of trigonelline, respectively.

Excretion of nicotinuric acid and trigonelline

Nicotinuric acid and trigonelline are the main excretory products derived from nicotinic acid. Therefore, in order to investigate whether they pass through the body completely and unchanged, they were administered separately to dogs on the low nicotinic acid diet. Each of three dogs received 100 mg. of trigonelline orally. Within 2 days, 75% had been excreted in the urine unchanged. In the next 2 days 10 to 15 mg. more of trigonelline could be accounted for. Two other dogs received subcutaneously 225 mg. nicotinuric acid (= 150 mg. nicotinic acid) and in 1 day excreted 95% of the nicotinuric acid as such. Since these dogs had been on a low nicotinic acid diet for 5 weeks, this complete excretion of nicotinuric acid showed that the dogs could not utilize nicotinuric acid in place of nicotinic acid, nor could they split it to form nicotinic acid. This is in harmony with the results of Dann and Handler ('41 c), who were unable to cure canine black tongue with nicotinuric acid given subcutaneously, and contrary to the finding of Woolley, Strong, Madden and Elvehjem ('38) in a single case of canine black tongue.

Tissue analysis of dogs on high nicotinic acid diet

We are grateful to Drs. W. J. Dann and P. Handler for analyses of the liver, kidney and muscle of dogs B, C and D, which were performed by their method ('41 a) as soon as the urine experiments on the high nicotinic acid diet were completed.

For normal dogs Dann and Handler ('41 a) found the following values (in micrograms per gram) for the nicotinic acid content of tissue: liver 90–233, mean 153; kidney 75–117, mean 95; muscle 59–80, mean 71. In the tissues of the three dogs saturated with nicotinic acid the following values were

obtained: liver 150, 178, 159; kidney 93, 93, 68; muscle 85, 88, 91 μg. per gram. Thus it was only in the muscles of these three animals that the nicotinic acid content was found to be above the normal range in each case, while the values for the liver and kidney fell well within that range, and indeed close to the normal means for these tissues. Since the muscles constitute the most extensive of the body tissues, the possibility of storage of nicotinic acid in them may be of some significance.

Black tongue dog

It is usually difficult to follow the urinary excretion of a dog exhibiting black tongue, since the onset of severe symptoms and of anuria is sudden. In some dogs, however, the progress of the disease is more gradual. One such dog of our series developed the mouth lesions and heavy salivation typical of the black tongue dog after 7 weeks on the low nicotinic acid diet. The dog remained moderately sick for almost 3 weeks during which time the daily excretion of NA and trigonelline was measured as shown in table 3. He suddenly became very

TABLE 3

Excretion of NA and trigonelline by a dog during the onset of black tongue and during dosage with nicotinic acid.

DAY	NA	TRIG.	DAY	NA	TRIG.
	mg.	*mg.*		*mg.*	*mg.*
50 [1]	0.4	1.9	71–72 [3]	1.5	0.5
51–52	0.4	1.6	73	1.9	1.1
53	0.6	1.8	74	1.1	2.7
54–55	0.5	0.7	75	1.4	7.4
56–57	0.7	0.8	76	2.9	19.4
58–59	0.5	0.7	77	1.2	6.6
60–61	0.9	0.6	78	1.3	15.9
62	0.6	0.6	79	1.0	9.3
63–64	0.4	0.3	80	1.1	15.3
65–66	0.4	0.2	81	0.8	15.6
67–68	0.4	0.1			
69–70 [2]	0.5	0.1			

[1] Mouth lesions and heavy salivation appeared in dog and persisted for the following 3 weeks.

[2] Dog became very sick. Severe necrosis and bloody diarrhea developed.

[3] Started to receive 25 mg. nicotinic acid subcutaneously each day.

ill on the seventieth day of the diet and was given 25 mg. of nicotinic acid subcutaneously each day, and the urinary NA and trigonelline determined as he recovered.

While the dog was sick, the NA excretion fell very little, but the trigonelline almost disappeared. The figures of 0.1 to 0.2 mg. of trigonelline reported in the table are too low to be reliable and may fall within the limits of error of the analytical method.

With the daily administration of 25 mg. nicotinic acid there was practically no increase in excretion of trigonelline until the fifth day. It then rose slowly and reached a level of about 15 mg. per day. During this period the excretion of NA did not rise significantly. The total nicotinic acid excretion, predominantly as trigonelline, resembled at the end that of normal dogs on a high nicotinic acid intake. The dog used or destroyed 10 mg. of nicotinic acid per day, which is similar to the 7 to 8 mg. per day unaccounted for on the high nicotinic acid diet, shown in table 2.

DISCUSSION

The evidence presented above definitely indicates the importance of trigonelline formation as an index of nicotinic acid metabolism in the dog. After a few days of starvation, there is a small drop in the trigonelline excretion of a healthy dog as the animal begins to conserve its body tissues. While on the low nicotinic acid diet, the trigonelline excretion continues to drop markedly, although NA excretion falls very little. When the dog develops black tongue, the urinary trigonelline disappears almost completely, and it is this cessation of trigonelline excretion which appears at the same time as the severe symptoms of black tongue. This could be due to lack of ability to make trigonelline or to lack of excess of nicotinic acid as the precursor. Since the ability to form trigonelline is recovered in a few days by administering nicotinic acid, it seems that the deficiency is only in the amount of nicotinic acid. However, the nicotinic acid content of tissues of black tongue dogs (Dann and Handler, '41 b) is not far enough below the normal to explain this unless

we assume that the larger part of the nicotinic acid is needed to carry on the functions of coenzyme as we know them now, and that a smaller part is necessary for an unknown, vital step in the body's total metabolism.

After 20 days on a low nicotinic acid diet, three doses of 25 mg. of nicotinic acid were required before the dogs excreted any substantial part of the 25 mg. dose. In the same way, the black tongue dog required 125 mg. of nicotinic acid before any of it was excreted, and it was only after a few more 25 mg. doses, that the proportion of the dose excreted resembled that of a normal dog. This response to dosage with nicotinic acid is the most accurate way of determining whether a dog is totally or partially deficient in nicotinic acid.

SUMMARY

A study of the excretion of acid hydrolyzable nicotinic acid derivatives (NA) and trigonelline in dogs, at different levels of nicotinic acid intake, is presented. Trigonelline and nicotinuric acid are the main end products of nicotinic acid metabolism found in dog urine.

The urinary excretion of trigonelline falls to 1.1 to 2.7 mg. per day on a low nicotinic acid diet, and to 0.1 mg. in the black tongue dog.

On the low nicotinic acid diet or in black tongue several 25 mg. doses of nicotinic acid are required before the dog will excrete part of the dose.

On a high nicotinic acid diet (1.1 mg. per kilogram) dogs excrete a total of about 7 mg. of the 12 to 15 mg. they ingest daily. Over 90% of this is in the form of trigonelline.

After saturation with nicotinic acid, doses of 100 mg. of this compound or nicotinamide are completely excreted in the form of trigonelline (75 to 94%) and nicotinuric acid (6 to 25%). The addition of glycine and choline does not influence the proportions excreted as nicotinuric acid or as trigonelline.

Neither trigonelline nor nicotinuric acid can be utilized by the dog.

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PANTOTHENIC ACID IN THE NUTRITION OF THE RAT[1]

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Lepkovsky et al. ('36) concluded that rats needed a "filtrate factor" which was distinct from what we recognize today as thiamine, riboflavin and pyridoxine. This filtrate fraction has been studied in relation to both the growth of rats and the prevention of graying in pigmented rats. Several earlier investigators (Hartwell, '23; Bakke et al., '30; and Gorter, '35) reported the production of hair depigmentation of black and piebald rats maintained on certain diets. Lunde and Kringstad ('38) and Morgan et al. ('38) observed both graying and retarded growth in black rats reared on synthetic diets. Oleson, Woolley and Elvehjem ('39) and Subbarow and Hitchings ('39) obtained growth responses by rats on adding pantothenic acid concentrates to synthetic rations similar to those used by the previous workers. Lunde and Kringstad ('39) concluded that the "filtrate factor" was distinct from the "anti-gray hair factor" since concentrates which promoted growth were not effective against hair depigmentation. Oleson, Elvehjem and Hart ('39) reported that pantothenic acid concentrates and other known vitamins were ineffective in preventing nutritional achromotrichia. Several

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investigators (Morgan and Simms, '40; Lunde and Kringstad, '40; Dimick and Lepp, '40; and Mohammad et al., '40) confirmed these results reporting that in addition to pantothenic acid, there is a second filtrate factor which is necessary for the maintenance of normal hair color in rats. The differentiation between these two filtrate factors was made before pure pantothenic acid was available and before the pantothenic acid requirement of rats was known.

György et al. ('40) were able to prevent and cure gray hair by feeding pantothenic acid concentrates and later György and Poling ('40 a) found synthetic calcium pantothenate to be active. Unna ('40), feeding synthetic calcium pantothenate, set the pantothenic acid requirement of the rat at 80 μg. per day and found that liver concentrates of known pantothenic acid potency were more effective in promoting growth than equivalent amounts of pure calcium pantothenate. Unna and Sampson ('40) were able to prevent and cure graying of the fur with 100 μg. of calcium pantothenate per day.

The complete maintenance of fur color has not been achieved by all investigators through the use of pure calcium pantothenate. Williams ('40) reported the failure of either pantothenic acid concentrate or synthetic calcium pantothenate to prevent or cure achromotrichia. Emerson and Evans ('41) have reported the prevention of pattern graying with calcium pantothenate but report that further supplementation is required to prevent "stippling." More complete prevention of graying was obtained by Dimick and Lepp ('40) when rice bran filtrate was used as a source of pantothenic acid than when pure calcium pantothenate was used. György and Poling ('40 b) were successful in curing the graying completely by feeding biotin when only a few scattered gray hairs remained after curing with pantothenic acid. Ansbacher ('41) was unable to prevent graying by supplying 500 μg. of calcium pantothenate per rat per day. Administration of p-aminobenzoic acid was effective against the symptoms he observed. Frost et al. ('41) reported a negligible effect of pantothenic acid alone against graying, but were able to get cures by

using liver extracts at levels which furnished as little as 40 μg. of pantothenic acid per day.

Somewhat over a year ago we attempted to perfect an assay procedure for the "anti-gray hair factor" which could be applied to naturally occurring materials. After many assays on animal tissues it was found that the activity of these samples paralleled very closely their pantothenic acid content as determined by the chick assay (Waisman et al., '39). When synthetic calcium pantothenate became available it was found to be effective against the graying we were observing. We wish to report in this paper our experiences with pantothenic acid in relation to both growth and graying in rats.

EXPERIMENTAL

The rats used for these studies were obtained from two sources, the black and piebald rats from our colony and the small number of albino rats used for growth studies from Sprague-Dawley. The rats were placed in individual screen bottom cages at 21 days of age and fed the ration ad libitum. The majority of the studies were conducted with basal ration 789 which consists of sucrose 73%, purified casein[2] 18%, salts IV[3] 4%, corn oil[4] 5%, thiamine 2 mg., pyridoxine 2 mg., riboflavin 3 mg., nicotinic acid 2.5 mg. and choline 1 gm. per kilogram. Two drops of halibut liver oil were given per week. The vitamins were added in solution to the casein and after drying the casein was ground with the sucrose and salts.

Synthetic calcium pantothenate[5] was fed daily in a supplement dish at the levels studied. All the rats were started on supplement at 21 days of age. On this basal ration the rats ceased to grow after 4 to 6 weeks and graying began to appear at about the same time. The gray hair sometimes appeared earlier in rats receiving low levels of calcium pantothenate than in those receiving the basal ration. However, spontaneous

[2] Labco from Borden and Company, New York City.
[3] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935).
[4] Mazola.
[5] Merck's.

cures were frequently observed when these animals were continued on these low levels (20–40 μg.) for several weeks.

Table 1 summarizes the results obtained with 140 black and piebald rats and 68 albino rats on basal ration 789, plus graded levels of calcium pantothenate. The growth results include both the albino and piebald rats.

TABLE 1

The effect of various levels of calcium pantothenate on growth and hair color of rats fed ration 789.

LEVEL OF CALCIUM PANTOTHENATE	NO. OF RATS USED	PERCENTAGE WHICH GRAYED	AVERAGE WEEKLY GAIN IN GRAMS FOR 6 WEEKS
μg.			
0	29	100	5.9
20	30	82	14.0
30	7	85	18.0
40	44	36	22.6
60	15	0	21.6
80	22	7	27.4
90	4	0	24.5
100	27	0	24.6
200	9	0	28.1
500	21	0	28.4

Rats receiving levels of calcium pantothenate above 40 μg. per rat per day showed no graying except in a few cases; at 40 μg. the results were variable with 36% graying, while at lower levels almost all rats turned gray. The requirement for growth agrees fairly well with the results of Unna ('40) although we obtained better growth on the optimum level. Some curative trials showed that definite signs of alleviation of graying occurred in from 3 to 6 weeks but usually about 4 weeks after the initiation of therapy (100–500 μg. per day). All rats were completely cured within a period of 8 weeks.

Studies with heated ration

The heated grain ration first introduced by Kline et al. ('32) is low in pantothenic acid. It has been used extensively in producing pantothenic acid deficiency in chicks, but has not been widely used for rats. Ansbacher ('40) reported

graying of rats on this ration when pyridoxine was added. We were able to cause graying of the fur of rats kept on the heated basal ration in 6 to 8 weeks. The ration consists of a basal mixture with the following percentage composition: ground yellow corn 58, wheat middlings 25 and crude casein 12 which were mixed and heated for 30 hours at 120°C., salts IV 4, and cod liver oil 1. To each kilogram of this mixture were added thiamine 2 mg., riboflavin 3 mg., pyridoxine 3 mg., nicotinic acid 25 mg., and choline 3 gm. Of twenty-three rats fed this basal ration only two did not become gray. Growth was slow, averaging 8.6 gm. per week for the first 6 weeks. Of the twenty-one rats receiving the basal ration to which had been added 10–20 mg. of calcium pantothenate per kilogram none showed signs of graying and the average growth for 6 weeks was 18.1 gm. per week.

Graying from other causes

Depigmentation of black and piebald rats has been reported (Gorter, '35; Keil and Nelson, '31; and Free, '40) on milk rations and attributed to copper deficiency. Free ('40) clearly differentiated between graying caused by a mineral deficiency and that caused by lack of an organic factor.

Graying was produced in piebald rats on whole milk supplemented with iron and manganese. The addition of copper at 50 μg. per day, as copper sulfate, completely prevented the condition but supplementation with 100 μg. of calcium pantothenate per day had no effect. The gray hair arising from lack of copper was clearly alleviated within 2 weeks after copper therapy was begun. The graying differed from that observed on basal ration 789 in that the hair had a definite brownish color instead of the silvery gray, observed in the vitamin deficiency.

Oettel ('36) reported graying of the fur of cats receiving hydroquinone. We attempted to repeat this observation using rats, but with no success. Rats receiving ration 789 plus adequate amounts of calcium pantothenate were fed 2–5 mg. of hydroquinone per day in aqueous solution. At the end of

5 weeks, growth was normal and there was no indication of graying. Raising the level of intake to 25–35 mg. of hydroquinone for 5 weeks produced no adverse effect on growth or hair color. Likewise on a milk ration no graying was noted when 10 mg. of hydroquinone was added to the mineralized milk each day. Martin and Ansbacher ('41) produced depigmentation in black mice by feeding a stock diet to which hydroquinone had been added. This was interpreted as a vitamin deficiency since p-aminobenzoic acid was effective in curing the gray hair. Although we used higher levels of hydroquinone our rats did not show the symptoms which the above workers noted in mice.

Following Ansbacher's ('41) report that p-aminobenzoic acid was effective against gray hair in rats which grayed while receiving as much as 500 μg. of calcium pantothenate per day, we fed this substance. It was not effective in relieving the gray hair noted in rats fed low levels (0–40 μg.) of pantothenic acid. When rats received adequate pantothenic acid a slight growth response was noted in rats from our colony. The average growth of four rats receiving ration 789 plus 80 μg. of calcium pantothenate was 19 gm. per week with a range of 16 to 22 while the average growth of rats receiving an additional supplement of 3 mg. of p-aminobenzoic acid was 27.8 gm. per week with seven of the eight showing a range of from 27 to 36 gm. per week. One rat grew at the rate of 17 gm. per week. Subsequent attempts to repeat this observation using rats from Sprague-Dawley have failed. With these animals ration 789 plus adequate pantothenic acid has given as high as 31 gm. per week growth, but added p-aminobenzoic acid has not increased growth. It should be pointed out that this might be due to the difference in strain or to previous nutritional history of the rats used. Thirty-one grams per week is better growth than we were able to obtain by feeding p-aminobenzoic acid to rats from our colony.

Balance studies

The large number of reports that natural crude concentrates were more effective in preventing gray hair than equiva-

lent amounts of pure pantothenic acid suggested the possibility of bacterial synthesis of pantothenic acid in the intestinal tract of the rats being studied. It seemed possible that these liver fractions were furnishing precursors or in some other manner stimulating synthesis of pantothenic acid which was being used by the animal. This, of course, would not explain the ineffectiveness of high levels of pure calcium pantothenate in preventing graying as some investigators had noted. It seemed unlikely that large amounts of pantothenic acid were available to the rats since animals on basal ration 789 often showed adrenal necrosis at death, a condition which is prevented by as little as 5 μg. of pantothenic acid per day (Mills et al., '40).

To test the possibility and to ascertain the requirement of the rat for pantothenic acid, balance studies were undertaken. The microbiological assay of Strong et al. ('41) was employed. Little quantitative data could be obtained on the pantothenic acid content of the feces, since added pantothenic acid was recovered to the extent of only 50 to 60%. However, the values as obtained indicated that considerable pantothenic acid was excreted in the feces. The amount was almost independent of the diet, since rats suffering from severe pantothenic acid deficiency excreted as much per gram of feces as rats receiving adequate pantothenic acid or liver fractions in which the pantothenic acid had been destroyed by autoclaving in acid solution.

The level of pantothenic acid in feces varied between 50 and 150 μg. per gram of air-dried feces as shown by this method or from 10 to 75 μg. per day. Corrected values would be approximately twice this high. The assumption that most of this pantothenic acid was synthesized by bacterial action in the cecum received support from the analysis of the contents of two portions of the intestinal tract. Three rats with severe pantothenic acid deficiency were used. The entire intestine from the stomach to the cecum contained from 1.5 to 5 μg. of calcium pantothenate, and the cecum and colon from 25 to 40 μg. It seems likely then that by far the greater part

of fecal pantothenic acid arises in the cecum and large intestine and may not be available to any appreciable extent to the rat.

Analysis of the urine was carried out simultaneously with fecal analysis. The urine was collected under toluene during 2–4 day periods and made up to a known volume by washing down the sides of the funnels. The microbiological assay was satisfactory, giving recoveries ranging from 89 to 116%[6] with an average value of 102%. Rats which had received basal ration 789 for several weeks excreted less than 1 μg. of calcium pantothenate per day, usually 0.3–0.4 μg. This amount was slightly less than that consumed in the ration, which contained about 0.2 μg. per gram. In no case was more pantothenic acid excreted in the urine than was consumed. This is another fact which makes utilization of pantothenic acid synthesized in the tract unlikely. Feeding various liver fractions did not increase urinary excretion above that noted when pure calcium pantothenate was fed at an equivalent level.

TABLE 2

Urinary excretion of calcium pantothenate in rats.

LEVEL OF INTAKE	NO. OF RATS	AVERAGE DAILY EXCRETION						AVERAGE
		1st wk.	2nd wk.	3rd wk.	4th wk.	5th wk.	6th wk.	
μg.		μg.	μg.	μg.	μg.	μg.	μg.	μg.
20	6	.67	1.0	.6	1.4	2.2	1.7	1.3
40	6	2.1	2.3	1.9	2.6	4.6	8.5	3.7
60	4	1.6	3.6	6.9	9.8	8.7	8.2	6.4
80	2	4.4	7.7	24.3	8.5	4.4	7.3	9.4
150	2	10.2	91.0	52.0	65.0	50.0	68.0	54.8

In order to ascertain the requirement of the rat, the urinary excretion of pantothenic acid was followed in rats receiving varying levels of calcium pantothenate, administered both orally and intraperitoneally. A summary of the results is given in table 2.

Since no differences were observed between the values obtained when the calcium pantothenate was given orally and when injected, no differentiation is made in the table. It will

[6] The calculations were based on single determinations and these values show normal variation for such determinations.

be noted that there is a general increase in excretion from the first to the sixth week which indicates a decrease in requirement as the rate of growth decreases. The results indicate a marked increase in the urinary excretion of pantothenic acid as the level of intake is raised from 80 to 150 μg. Up to 80 μg. the excretion is very low, and at 150 μg. about 50 μg. are excreted indicating that 100 μg. is approximately the requirement for the growing rat. This is in good agreement with the growth experiments reported in a previous section.

DISCUSSION

It is evident from the data presented in the paper that rats on diets containing only the synthetic forms of the B vitamins require 80 to 100 μg. of calcium pantothenate per day for maximum growth. The actual requirement cannot be much higher than this value since a growth rate of 24 to 28 gm. per week is obtained. However, the requirement may be found to be lower in the presence of all essential factors.

An increase in the rate of growth of rats fed synthetic rations containing all the known vitamins has been obtained by adding liver extracts (Unna, '40; Elvehjem et al., '41). This growth increment may be small but it is always definite and indicates that these extracts contain additional factors required by the rat. However, successful reproduction has been reported by Jukes ('40). We have been able to raise rats through four generations with no pronounced difficulty on ration 789 together with 2 mg. calcium pantothenate per 100 gm. ration and α-tocopherol at 1 mg. per week. In all cases the rate of growth was not optimum but the young finally reached maturity and reproduced. Some of the mothers showed impaired lactation especially with the first litter.

In much of the earlier work the pantothenic acid requirement was assumed to be about 30 μg. per day. No increment in the rate of growth was obtained by raising the level from 30 μg. to higher levels. In our laboratory the lack of response was due largely to an inadequate supply of choline. The present studies, as well as those reported by Unna, show that

30 μg. may give fair growth but does not give the optimum rate or protection from graying. Levels of 40 μg. per day frequently caused graying to occur faster and to a more extensive degree than when no pantothenic acid was given. These facts may account for the earlier reports that pantothenic acid preparations were ineffective in the prevention of graying.

The fact that pantothenic acid alleviates hair depigmentation under the conditions of our experiments does not mean that this vitamin is the only organic factor concerned with the normal hair color. It is possible that different rations may give various deficiencies because of their effects on the intestinal flora. Studies on this problem are now in progress in this laboratory (Black et al., '41).

SUMMARY

Rats fed a synthetic ration deficient in pantothenic acid became gray in 4–6 weeks. Levels of calcium pantothenate above 40 μg. per day prevented or cured this condition. The rate of growth paralleled the level of pantothenic acid intake up to 80 μg. per day. Studies on the urinary excretion of this vitamin indicated that the daily requirement for the growing rat is approximately 100 μg. of calcium pantothenate per day.

Rats fed a heated grain ration showed graying which was likewise prevented and cured with synthetic calcium pantothenate. Copper deficiency caused a graying which did not respond to pantothenic acid but was rapidly cured by administration of copper.

Feeding hydroquinone failed to produce graying in rats receiving synthetic or milk rations. p-Aminobenzoic acid was entirely ineffective against the graying produced in rats by feeding a synthetic ration under our experimental condition.

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CRITERIA OF RESPONSE IN THE BIO-ASSAY OF VITAMIN E[1]

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Only during the past few years has any serious effort been made to improve the procedure for the bio-assay of vitamin E, based upon prevention of fetal resorption in the female rat, as established by Evans and Burr in 1927. Through proper regulation of the diet of breeding rats whose offspring are to be used for bio-assay purposes, it has been possible to dispense with the necessity of demonstrating sterility by means of a proven resorption and to use the first pregnancy period of the vitamin E deficient female for assay tests (Ringsted, '35; Bacharach et al., '37, '38; Mason and Bryan, '38, '40). This has greatly reduced the time and labor involved in the preparation of rats for bio-assay and for other experimental purposes. There still exists an unfortunate lack of uniformity regarding the duration of feeding of the test dose of vitamin E, and the most satisfactory means of evaluating the response obtained. The present discussion deals primarily with the latter problem.

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According to generally accepted criteria, a positive response implies the delivery of one or more progeny by a rat which otherwise would have undergone a resorption gestation. Uncertainty which may arise through cannibalistic proclivities of the mother can be avoided, in large part at least, by placing the parturient mother upon a raised coarse-screen floor such that the offspring fall out of reach of the mother soon after delivery. A negative response necessarily implies the intrauterine resorption of all implanted embryos. Methods for evaluating this "all or none" type of response as applied to bio-assay tests have been extensively discussed in recent papers by Bacharach and Allchorne ('38) and Bacharach ('38, '39).

Since the number of young delivered by rats showing a positive response will vary from one to many, and since negative responses must include uterine states varying from early resorption of fetal sites to those just falling short of a completed gestation, it is obvious that there exists a definite variation in the intensity of response of either type. No method has yet been devised for evaluating the latter. The efforts of Palmer ('37) to assess responses on the basis of data obtained at parturition, correlated with those obtained at autopsy immediately after delivery or after resorption, represent a step in this direction, even though the results offered no final solution to the assay problem. The studies presented in the present report represent an attempt (1) to establish more satisfactory criteria of response based upon data obtained at laparotomy or at autopsy on the sixteenth day of pregnancy, and (2) to determine whether such data can best be evaluated in terms of the "graded" or the "all or none" type of response.

EXPERIMENTAL

For use in the bio-assay tests reported in these studies, virgin E-deficient rats were standardized according to the following procedure. Rats of the breeding colony were reared and maintained on a diet of commercial dog biscuit known

to afford approximately four times the minimal daily requirement of vitamin E. Between the twelfth and fourteenth days of lactation the mother and offspring were transferred to the vitamin E deficient diet (casein, 20%; cornstarch, 48%; lard, 18%; salts, 4%; brewers' yeast, 8%; cod liver oil, 2%) upon which the progeny were subsequently maintained after weaning on the twenty-first day of lactation. The latter were mated with normal males when they had attained body weights of 150 gm. or better, usually within 30 to 50 days after weaning, and were used for assay tests during their first pregnancies. Evidence concerning the uniform and critical level of vitamin E depletion in such rats, and the factors involved in attaining this end, has been presented in earlier reports (Mason and Bryan, '38, '40; Mason, '40).

RESULTS

Gradations in positive and negative response. The results of a series of bio-assays designed to test the uniformity of response of our standardized virgin rats (table 1) confirm what has been stated above concerning the relationship which may exist between the size of the dose of vitamin E given

TABLE 1

Summary of bio-assay of a concentrate of vitamin E,[1] showing gradations in positive responses with increasing doses.

SINGLE DOSE IN MG.	NO. OF RATS USED	NO. RESORBED[2]	NO. DELIVERED	AVER. NO. OFFSPRING PER LITTER	NO. OF LITTERS WEANED[3]
3.75	5	5	—	—	—
5.0	10	8	2	2.5	—
6.25	10	6	4	3.3	—
7.5	10	2	8	3.4	2
10.0	11	1	10	6.6	3
15.0	11	—	11	6.9	8

[1] Supplied through the kindness of Dr. H. A. Mattill, Department of Biochemistry, State University of Iowa, Iowa City. Administered as a single dose to E-deficient rats on the first day of their first pregnancy.

[2] All resorptions were verified by autopsy.

[3] All litters showed vitamin E deficiency paralysis during the last few days of lactation.

and the intensity of the positive responses obtained, i.e., the number of young delivered at term. It is also worthy of note that the amount of vitamin E required for successful lactation was at least several times the minimal requirement for delivery at term, an observation which we have repeatedly confirmed (see also table 4).

During the course of another series of assay tests forty rats were subjected to laparotomy, and eighty others to autopsy, at different stages of pregnancy. A wide range of variation was noted in the gross appearance of the uteri in rats which, according to accepted criteria, would be regarded as exhibiting a negative response. Data obtained relative to size and state of the implantation sites, and weights of the uteri and their contents, suggested that the most satisfactory criteria for evaluating gradations in both negative and positive responses might be obtained by resorting to autopsy of all rats on the sixteenth day of pregnancy, at which time the body weights and uterine weights of untreated controls usually reached a maximum. On the basis of this experience the following procedure, briefly discussed in an earlier report (Mason, '39), was carried out in 600 routine bio-assay tests. At autopsy on the sixteenth day of pregnancy the cervix was severed at its mid-point and the full uterus, freed of mesentery and ovaries, was carefully weighed. After recording the live fetuses,[2] dead fetuses and resorptions the uterus was completely emptied and weighed. From these data two factors were obtained: (W) the weight of the uterine contents, and (N) the number of viable fetuses, when two or more were present. The presence of a single viable fetus, or of one or more dead fetuses, was disregarded except that their existence was reflected in the increased weight of the uterine contents. This practice was based upon the assumption that a single fetus surviving to the sixteenth day in a uterus in which resorption or recent fetal death had

[2] The combined weight of viable fetuses, together with intact membranes and placentae, varied from 0.92 to 1.34 gm. and averaged 1.145 gm. for 1222 fetuses weighed. The average weight of 160 fetuses of stock control rats at the sixteenth day was 1.248 gm.

occurred at other implantation sites had little likelihood of being delivered at term; this question is further discussed below. An arbitrary distinction was made between resorption sites and dead fetuses, depending upon whether the total weight of the fetal remnants and placenta was less, or greater, than 0.4 gm. Dead fetuses, even when approximating normal fetuses in size, were readily distinguished by their waxy white color and the pallor of the heart and liver, as well as by the absence of circulation in the blood vessels of the yolk sac, when examined with a dissecting microscope.

Attempts to evaluate gradations in response. After an extensive analysis of the data obtained, the formula, $\frac{W+N}{5}$ = uterine index, was adopted as the best means of numerically expressing the gradations in response observed. In the application of this formula to 600 routine bio-assay tests (table 2) it was found that responses were generally divisible into "negative", "subminimal" and "positive", based on uterine-index values less than 0.35, 0.35 to 0.99 and 1.0 or greater,

TABLE 2

Relationship between the number of live and dead fetuses and the distribution of uterine indices in 600 rats used in routine bio-assay tests and autopsied on the sixteenth day of pregnancy.

GROUP[1]	FETUSES PRESENT		DISTRIBUTION OF UTERINE INDICES					
			<.35 NEGATIVE		.35–.99 SUBMINIMAL		>.99 POSITIVE	
	Live	Dead	No. of rats	Range of indices	No. of rats	Range of indices	No. of rats	Range of indices
A	0	0	203	(.02–.34)	8	(.36–.46)	—	—
B	0	1	10	(.21–.34)	12	(.36–.60)	—	—
	0	2	—	—	12	(.38–.84)	—	—
	0	3 or >3	—	—	6	(.63–.97)	3	(1.06–1.51)
C	1	0	3	(.70–.32)	12	(.35–.61)	—	—
	1	1 or >1	—	—	18	(.40–.97)	1	(1.27)
D	2	0	—	—	4	(.87–.98)	6	(1.0 –1.25)
	2	1 or >1	—	—	—	—	17	(1.13–1.65)
	3 or >3	±	—	—	—	—	285	(1.0 –5.65)

[1] The animals are grouped on the basis of degree of response, irrespective of dosage. Hence, no special significance is attached to the fact that the groups are arranged, in general, in order of increased dosage.

respectively. Untreated rats (negative controls), not included in the data of table 2, invariably gave values less than 0.35. It is evident that many responses which would be classed as negative on the basis of data obtained at the end of pregnancy can be recognized as subminimal by means of the uterine-index values or, in many instances, merely by gross inspection of the uterus at the sixteenth day of gestation. Likewise, the presence of dead fetuses in utero at this time, regardless of the absence or presence of viable fetuses, indicates a critical level of response. On the other hand, in the evaluation of graded responses, especially in rats receiving critical levels of dosage, the usefulness of the index values becomes impaired by the uncontrollable fluctuation of numerical values in the positive range primarily due to variations in the total number of implantation sites in different test animals (table 3). To a lesser extent, variations in the weight

TABLE 3

Showing the variation in total number of implantation sites (live fetuses, dead fetuses and resorption sites combined) observed in 1500 rats used in bio-assay tests and examined by laparotomy or autopsy at the sixteenth day of pregnancy.

TOTAL NUMBER OF IMPLANTATION SITES	NO. OF RATS	% OF RATS	TOTAL NUMBER OF IMPLANTATION SITES	NO. OF RATS	% OF RATS
1	14	0.93	8	239	15.93
2	18	1.2	9	351	23.4
3	19	1.27	10	319	21.27
4	23	1.53	11	193	12.87
5	36	2.4	12	66	4.4
6	55	3.67	13	35	2.33
7	120	8.0	14 or more	12	0.8

of the fetuses and annexa secondary to age differences of a few hours at this period of rapid growth may affect the values. In turn, these fluctuations overbalance the numerical gradations of subminimal responses. Numerous modifications of the uterine index formula designed to minimize the variables mentioned have offered no better solution to the problem of evaluating gradations in response.

In a series of bio-assay tests involving groups of eighteen to twenty rats at four different levels of E-dosage, it was found that the average of the uterine indices for each group when plotted against the dosage levels gave essentially the same curve as obtained by plotting in a similar manner the fertility rate; i.e., the per cent of rats in each group giving a positive response, as determined by the presence of two or more viable fetuses at the sixteenth day of pregnancy. As pointed out below, the latter criterion of positive response and that based upon parturition are virtually interchangeable. It thus appears that the uterine-index procedure offers no particular advantage over the customary method of treating the response as an "all or none" type and expressing the results in terms of fertility rate, as proposed by Bacharach and Allchorne ('38).

Criteria of response obtained at the sixteenth day of pregnancy. In evolving the uterine index discussed above, it was assumed that a single fetus surviving among three or more resorption sites[3] at the sixteenth day had little likelihood of being delivered at term; whereas at least one of two or more viable fetuses would undoubtedly be delivered. This postulate was based upon a preliminary series of sixty-two bio-assay tests on paired littermates, one of each pair being autopsied at the sixteenth day and the other allowed to continue to term. When positive responses were based upon the presence of at least two living fetuses at the sixteenth day and upon the delivery of at least one at term, respectively, the proportion of negative and positive responses was essentially the same (table 4).

In order to obtain more direct evidence on this point, 215 rats used in routine bio-assay tests were laparotomized on the sixteenth day of pregnancy. Complete resorption was

[3] In concurrence with a suggestion made to the writer by Dr. L. S. Palmer, it seems advisable to exclude from the computation of bio-assay results all rats which possess less than four implantation sites, regardless of whether the latter are represented by resorptions or fetuses. The number of animals eliminated from tests by adhering to this rule is not great, amounting to but 3.4% in the 1500 rats represented in the data of table 3.

TABLE 4

Paired bio-assay tests of a concentrate of vitamin E, comparing criteria of positive response based upon (1) delivery of one or more offspring at the end of pregnancy, and (2) the presence of two or more viable fetuses at the sixteenth day of pregnancy.

DOSE IN MG.[1]	RATS CONTINUED TO TERM		RATS AUTOPSIED ON SIXTEENTH DAY OF PREGNANCY		
	No.	Result	No.	Result	Implantation sites expressed as Living-Dead-Resorptions[2]
10	3	3 neg.	3	3 neg.	0-0-10; 0-0-10; 0-0-9
15	4	4 neg.	4	4 neg.	0-0-6; 0-0-11; 0-0-13; 1-1-3
20	9	7 neg. 2 pos.[3]	9	5 neg. 4 pos.	0-0-10; 0-0-9; 0-1-6; 1-1-7; 1-5-4 4-1-6; 7-2-1; 7-0-2; 8-0-2
30	9	2 neg. 7 pos.[4]	9	2 neg. 7 pos.	1-0-8; 1-3-5 2-1-14; 4-0-6; 4-0-6; 7-0-0; 7-2-0; 8-0-2; 9-0-0
40	6	6 pos.[5]	6	6 pos.	2-2-6; 5-0-4; 7-0-1; 8-0-2; 8-1-2; 9-0-1

[1] Fed as a single dose on the fourth day after positive mating.

[2] This method of recording the response conveys a convenient picture of the uterine state at autopsy. It should be stated, however, that occasional resorption sites are of normal occurrence in rats receiving an adequacy of vitamin E. Of seventy-five normal stock rats of the breeding colony autopsied on the sixteenth day of pregnancy, forty-two possessed one or more sites of advanced resorption interspersed between normal fetal sites.

[3] Only one offspring in each litter. Both died within 2 days.

[4] Average of 5.7 young per litter, none of which survived more than 4 days.

[5] Average of seven young per litter. Five litters successfully weaned, but paralysis of young appeared in all instances.

found in 103, and one or more viable fetuses [4] encountered in the remaining 112 which were continued to term and placed upon an elevated screen to permit the newborn to fall out of reach of the mother as delivered. Of seventy-two rats possess-

[4] It was sometimes difficult to determine at laparotomy whether or not certain fetuses were viable, even though both uterine horns were routinely withdrawn through the incision and carefully examined. Unless the operator was convinced that death had occurred, the fetuses were classed as living.

It is worthy of note that none of these rats, and none of several hundred other rats coming to delivery after receiving critically low dosage of vitamin E at various intervals prior to or during pregnancy, has exhibited the prolongation of gestation described by Barrie ('38) in rats under similar conditions and ascribed by her to impaired function of the corpus luteum secondary to anterior pituitary deficiency. Parturition invariably occurred between the twenty-first and twenty-third day, irrespective of the viability of the fetuses in utero. Since this is also the experience of many other investigators, the gestational abnormalities observed by Barrie can best be attributed to some experimental factors other than an inadequacy of vitamin E.

ing more than four viable fetuses at laparotomy, fifty delivered an equivalent number at term; while twenty-two delivered fewer (usually one or two) than were present at operation. Of the remaining forty rats, with one to four live fetuses at laparotomy (table 5), twenty would also be positive by either

TABLE 5

Record of forty rats used in E-assay tests and possessing less than five viable fetuses on the sixteenth day of pregnancy, showing the distribution of live fetuses-dead fetuses-resorption sites at laparotomy and the number of offspring delivered at term (indicated in parentheses).

RAT NO.	UTERUS	RAT NO.	UTERUS	RAT NO.	UTERUS	RAT NO.	UTERUS
2158	4-0-1 (4)	2668	2-1-2 (2)	2735	2-1-6 (0)	2862	1-0-5 (0)
2702	4-0-2 (4)	2813	2 1-4 (2)	2983	2-2-0 (0)	3032	1-0-7 (0)
2739	4-0-7 (4)	2822	2-1-5 (2)	2686	2-2-8 (0)	2670	1-1 9 (0)
3004	4-2-0 (4)	2715	2-0-2 (2)	2754	2-3-3 (0)	2741	1-2-4 (0)
2595	4-2-1 (3)	2740	2-0-2 (1)	2964	2-3-3 (0)	2838	1-2-5 (0)
2847	4-0-2 (3)	3001	2-0-5 (1)	2687	1-0-4 (1)	3049	1-2-4 (0)
2605	4-1-4 (2)	2988	2-0-7 (1)	2714	1-0-8 (1)	2743	1-3-5 (0)
2616	4-1-5 (2)	2691	2-1-7 (1)	3050	1-1-2 (1)	2872	1-3-5 (0)
3042	3-2-2 (3)	2969	2-2-4 (1)	2581	1-0-0 (0)	2894	1-3-4 (0)
2562	3-2-4 (2)	2700	2-5-3 (1)	2709	1-0-1 (0)	2717	1-4-6 (0)

criteria and twelve others negative, leaving eight animals whose responses differ when measured by the two criteria. These comprise five (out of fifteen) which failed to deliver either of two viable fetuses, and three (out of fifteen) which delivered a solitary fetus, present at the time of operation. Since these eight animals represent but 7% of the 112 with viable fetuses at laparotomy, and only 3.5% of the 215 rats operated upon after receiving vitamin E dosage close to the threshold level, it may be assumed that the two criteria are virtually equivalent in evaluating the "all or none" type of response in routine bio-assay tests.

Advantages of the 16 day autopsy procedure. On the other hand, the state of the uterus at the sixteenth day of pregnancy more clearly reveals the nature of individual responses, especially those of the subminimal type, than does the information obtained at the end of pregnancy. This shortened assay procedure possesses other advantages which warrant brief comment: first, a reduction of about 6 days in the time required

for completion of assay tests (of particular value in preliminary or exploratory types of assays) with the resultant saving in diet and animal care, together with the economy in cage space, since assayed rats can be grouped instead of being caged individually for data on delivery; second, the opportunity to observe the actual state and number of implantation sites, permitting the elimination of rats with less than four implants and the recognition of sub-minimal types of response. Although there seems to be no alternative but to consider the latter as negative in the final evaluation of assay results, they afford valuable and trustworthy indicators of a critical level of dosage. The same may be said of positive responses associated with the presence of dead fetuses at the sixteenth day.[5] A third advantage lies in the prompt detection of states of pseudopregnancy with bleeding[6] which, unless vaginal smears are followed daily, can easily be misinterpreted as resorption pregnancies and incorrectly recorded as negative responses unless the uterus is inspected during gestation or soon afterward. The usefulness of the abbreviated bio-assay procedure proposed is exemplified by a series of studies presented elsewhere (Mason, '42).

[5] A separate report will deal more specifically with a hitherto undescribed hemorrhagic state which I have frequently observed in both dead and living fetuses of low-E mothers. Since the incidence of this hemorrhagic state increases as the dosage of vitamin E is decreased, and since it cannot be prevented by administering relatively large doses of vitamin C or vitamin K to the mother, there is good reason to believe that it is a pathognomic sign of vitamin E deficiency and the cause of fetal death during late pregnancy. Its occurrence in association with positive assay responses constitutes a helpful indicator of critically low levels of dosage.

[6] Although various investigators have noted the frequent occurrence (10%–15%) of pseudopregnancy in rats mated for vitamin E assays, no particular reference has been made to the vaginal bleeding sometimes associated with this state. This latter phenomenon is characterized by the appearance of blood in the vaginal smear 1 or 2 days prior to the end of the 10- to 17-day period of pseudopregnancy. Autopsy at the onset of bleeding consistently reveals one or more small deciduomata which, in the course of their regression, give rise to the blood observed. This undoubtedly corresponds to the condition described by Evans ('28) as occurring in 52% of E deficient rats, and in but 3% of normal rats, made pseudopregnant by mating with vasectomized males. The frequency of bleeding in my pseudopregnant E deficient rats has been much less (about 5%) than observed by Evans.

SUMMARY AND CONCLUSIONS

1. Attempts to evaluate recognized gradations in negative and positive responses to vitamin E administration, based upon the number of live fetuses and the weight of the uterine contents at the sixteenth day of pregnancy in 600 rats used in assay tests, gave results not much superior to those obtained by treating responses as an "all or none" type, except for the recognition of a sub-minimal type of response otherwise classed as negative.

2. On the other hand, the presence of two or more viable fetuses in utero at the sixteenth day of pregnancy was found to afford a criterion of positive response essentially equivalent to, and in certain instances more equitable than, that based upon delivery of progeny at term.

3. In addition to advantages arising from the shortening of the test period for bio-assay, with the resultant economy of diet and animal care, examination of the uterus at the sixteenth day gives information especially pertinent to critical levels of response and prevents misinterpretation of certain atypical uterine states as negative responses to dose.

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DISTRIBUTION OF VITAMIN E IN THE TISSUES OF THE RAT[1]

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Knowledge concerning the distribution and storage of vitamin E in the animal body is decidedly limited. The rat bio-assay test is laborious and chemical methods have failed to give consistent results when applied to the detection of the vitamin in tissues. The pioneer studies of Evans and Burr ('27), in which the rat bio-assay test was applied to tissues from rats, cattle, pigs and sheep, indicated that vitamin E is widely distributed in the animal body but is never present in very great concentration in any one organ or tissue. Musculature, body fat and liver, in the order named, contained appreciably more vitamin than other tissues. In like manner, vitamin E has been demonstrated in the milk, musculature and adipose tissue of goats fed a normal diet, but not in the tissues of goats reared on a diet deficient in the vitamin (Underbjerg, Thomas and Cannon, '39). The data presented in these reports cannot be treated quantitatively, due to diversity in the vitamin E intake of the donor animals and to variations in the time of feeding of the test animals.

The various chemical tests for vitamin E have not given harmonious results when applied to the quantitative estimation of the vitamin in vegetable oils and concentrates of the latter (Smith and Bailey, '39). Uncertainty as to the speci-

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ficity of the reactions in the presence of many interfering substances has greatly restricted their application to animal tissue. The fact that such methods permit no discrimination between α, β, and γ tocopherols, which differ somewhat in their biological activity, prevents close correlation between chemical and biological evaluations of vitamin E. The spectroscopic method has demonstrated tocopherols in the body fat, muscles, urine, feces and blood of rats previously fed sources of the vitamin, but has revealed none in the liver or other tissues, suggesting that the tocopherol molecule is readily broken down or ineffectively stored by the organism (Cuthbertson, Ridgeway and Drummond, '40; Moore and Rajagopal, '40). Furthermore, results obtained by spectroscopic and colorimetric methods are not always in close accord. On the other hand, Karrer, Jaeger and Keller ('40) reported the presence of considerable tocopherol in the liver and musculature of horses and cattle as demonstrated by colorimetric and by potentiometric methods, the two procedures giving comparable results.

In view of the inadequacies of chemical means for evaluating vitamin E, any reinvestigation of its distribution in the animal body entails the use of the longer bio-assay method. Certain modifications of the latter have increased its reliability and permitted a significant shortening of the assay period (Mason, '42). These improvements have greatly facilitated the studies presented in this report, which are concerned with the biological estimation of vitamin E in organs and tissues of rats maintained on three different levels of vitamin E intake. The rat was selected as a basis for study since its need for vitamin E is indisputable and its minimal daily requirements reasonably well established for both sexes (Mason, '40). It was hoped that the results obtained from such a study might supplement existing knowledge concerning the metabolic utilization of the vitamin and, at the same time, afford a standard for testing the reliability of chemical methods for detecting its presence in organs and tissues.

EXPERIMENTAL

The three groups of rats from which tissues were obtained for bio-assay tests will be referred to as: (1) "low-E" rats, reared on a commercial dog food;[2] (2) "high-E" rats, fed for a period of 2 to 4 months on a diet of fresh, raw wheat germ supplemented with a small amount of casein and inorganic salts; and (3) "excess-E" rats, reared to 3–4 months of age on a diet containing 5% of mixed tocopherols.[3] It was estimated that the daily intake of vitamin E for the three groups was approximately 4, 100 and 10,000 times the minimal daily requirement, respectively.

Organs and tissues obtained from the donor rats were trimmed, weighed, placed in glass vials, frozen and stored in a refrigerator at 0°C., except when removed long enough to obtain portions for feeding. The tissues were usually fed within 2 weeks following their removal from animals, although there was no indication that longer periods of storage resulted in any loss in vitamin E content. Body fat was mixed with a casein-cornstarch-yeast mixture in order to be effectively administered. Blood was fed in the same manner, or as a concentrated alcohol-ether extract.

Standardized E-deficient females, prepared as described elsewhere (Mason, '42), were mated when weighing from 150 to 175 gm. and fed the tissues to be assayed during the first to tenth days of their first pregnancy. Aliquot parts of the total dose, the number varying with the size of the latter, were fed as daily doses about equally distributed around the fifth day of pregnancy.[4] The E-deficient diet was removed 3 to 6 hours before the test substance was fed, and a similar

[2] "Wayne Blox", manufactured by Allied Mills, Inc., Chicago.

[3] I am indebted to Dr. P. L. Harris of Distillation Products Inc., Rochester, N. Y., for his kindness in providing these rats. Due to the limited number of animals available, assays were restricted largely to liver and muscle.

[4] This procedure was followed on the basis of evidence (unpublished studies) indicating that single doses of vitamin E become increasingly more effective the later they are administered during the first 8 days of pregnancy. Throughout these studies the day following that on which sperm were found in the vaginal smear, or a vaginal plug observed, is referred to as the first day of pregnancy.

TABLE 1

Results of bio-assay tests showing the distribution of vitamin E in the rat.

AMT.	DA. FED	UTERUS [1]
	Liver (low E)	
140	1–10	5–1– 1
120	3–10	5–0– 3
120	3– 9	8–0– 1
120	3– 9	0–0–10
120	2– 8	0–0– 8
110	2– 8	9–0– 3
110	3– 9	0–0– 9
110	3– 9	0–0–10
100	3– 8	4–3– 2
100	3– 8	0–0–10
100	3– 8	0–0–11
100	3– 8	0–0–11
80	3– 7	0–0– 8
80	3– 7	0–0– 9
60	2– 7	0–0–10
50	3– 7	0–0–10
50	3– 7	0–0– 9
40	4– 6	0–0– 9
30	5– 6	0–0–15
20	4– 6	0–0– 7
	Liver (high E)	
40	1–10	7–0– 0
30	1– 5	9–0– 0
30	4– 6	9–0– 1
20	3– 5	9–0– 0
15	5	10–0– 0
10	5	9–0– 3
10	5– 6	8–0– 1
10	5– 6	8–0– 0
10	5– 6	9–1– 0
10	5– 6	0–0– 9
10	4– 6	0–0– 8
7.5	5	9–0– 0
7.5	5– 6	8–1– 1
7.5	5– 6	0–1– 5
7.5	5– 6	0–0– 9
7.5	5– 6	0–0– 9
7.5	7– 8	0–0– 9
7.5	5– 6	0–0– 9
7	5	0–0– 7
6	5	0–0–12
5	5	2–1– 2
5	5– 6	1–3– 5
5	5– 6	0–0– 8
5	5	0–0–10
5	5	0–0–11
3	5– 6	0–1– 5
3	5– 6	0–0– 7
3	5	0–0–10
	Muscle (low E)	
100	1– 9	2–1– 4
80	1– 8	2–1– 5
70	3– 9	5–1– 1
70	3– 7	8–0– 1
70	3– 7	0–0– 2
70	3– 7	0–0– 5
60	3– 7	7–1– 2
60	3– 6	4–1– 5
60	4– 8	2–3– 2
60	4– 8	5–4– 0
60	4– 8	7–0– 1
60	4– 8	0–0– 6
60	3– 5	0–0–10
60	4– 8	1–0– 7
50	4– 6	0–1– 8
50	4– 7	0–0– 8
50	4– 7	0–0– 8
40	4– 7	0–0– 8
40	3– 6	0–0–10
	Muscle (high E)	
60	1– 9	6–0– 2
60	1– 7	10–0– 0
40	5– 7	4–0– 1
40	4– 6	9–0– 0
30	4– 7	7–0– 0
30	4– 6	8–0– 1
20	4– 6	8–0– 0
20	6– 7	10–0– 0
20	4– 6	7–0– 4
20	4– 5	0–0–10
15	4– 5	7–0– 1
15	6– 7	0–2– 8
15	6– 7	0–0–11
15	6– 8	2–2– 0
15	5– 7	2–0– 7
10	7	0–1– 7
10	6	0–0– 6
10	4	0–0– 1
7	6	0–0– 7
	Kidney (low E)	
80	3– 9	9–3– 1
60	2– 8	3–1– 6
60	4– 8	4–2– 2
60	4– 8	0–0–10
60	3– 7	0–0–10
50	4– 8	0–0– 9
45	4– 7	3–5– 1
40	4– 7	0–0– 9
40	4– 6	0–0– 9
30	5– 8	0–0– 9
	Kidney (high E)	
20	3– 5	9–1– 0
20	4– 6	3–0–10
20	4– 6	0–0–12
15	5– 7	10–1– 1
15	4– 6	7–2– 1
15	4– 6	5–2– 2
15	4– 6	3–0– 7
15	3– 5	0–0– 9
10	5– 7	6–0– 3
10	5– 6	0–0– 9
10	5– 6	0–0–10
10	6– 8	0–0– 8
10	4– 6	0–0–10
	Body fat (low E)	
50	6–13	10–0– 1
45	1–10	1–1– 8
40	1–10	0–1– 8
30	2–12	0–0–11
30	1–10	0–0–11
20	1–10	0–0– 6
20	0– 7	0–0– 9
	Body fat (high E)	
35	1– 9	9–0– 0
30	1–10	6–0– 4
20	2– 8	10–0– 0
20	1–10	10–0– 0
20	1–10	6–0– 0
20	3–10	6–0– 2
15	3– 8	7–1– 3
15	3– 8	0–1– 7
15	3–10	0–0– 9
15	5–10	0–0– 8
15	5–10	0–0– 9
15	1– 7	0–0–10
10	5–10	0–0– 6
10	5– 9	0–0–10
10	5–10	0–0– 8
10	4– 5	0–0– 9
	Pancr. and Thym. (low E)	
40	4– 7	6–0– 4
40	4– 7	0–0– 8
30	4– 7	0–0– 9
30	3– 7	0–0– 8
	Pancr. and Thym. (high E)	
30	4– 6	7–0– 1
13	5– 7	9–0– 0
	Heart (low E)	
40	4– 7	8–0– 2
40	5–10	7–0– 2
30	5– 7	9–0– 1
30	4– 6	0–0– 9
30	4– 7	0–0– 4
27.5	4– 7	0–0– 7
10	4	0–0–11
	Heart (high E)	
15	4– 6	5–0– 1
10	5– 7	8–0– 1
10	4– 6	2–5– 3
10	7	2–0– 2
10	5– 7	0–0–11
8	5– 6	0–0–12
7.5	5– 6	0–3– 6
	Lung (low E)	
45	4– 8	7–1– 1
40	4– 7	8–0– 0
40	4– 7	7–0– 0
40	3– 6	6–3– 2
35	4– 7	0–1– 7
35	4– 7	0–0– 7
30	4– 7	1–2– 4
30	5– 7	1–1– 2
30	4– 6	0–1– 9
30	4– 7	0–0– 9
30	4– 7	0–0– 8
	Lung (high E)	
15	7	11–0– 0
15	4– 6	9–0– 0
15	4– 6	8–0– 0
10	4– 6	11–0– 0
10	6– 7	6–1– 1
10	5– 6	4–0– 2
7.5	5– 7	7–0– 2
7.5	5– 6	5–0– 3
7.5	6– 7	0–0–12
7.5	5– 6	0–0–10
7.5	5– 6	0–0– 8
5	5– 6	0–0–13
	Spleen (low E)	
35	5– 8	8–0– 0
35	3– 7	2–2– 4
35	4– 7	1–2– 5
32	5– 8	8–1– 3
30	5– 8	8–0– 0
30	4– 7	7–2– 3
30	4– 6	2–0– 5
30	3– 7	0–0– 8
	Spleen (high E)	
25	4– 9	2–0– 0
17.5	4– 6	9–0– 0
14	5– 7	9–0– 0
7.5	6– 7	0–0– 3

TABLE 1 (*continued*)[1]

AMT.	DA. FED	UTERUS
	Testis (low E)	
60	4– 8	7–1– 2
60	4– 8	2–0– 6
60	4– 8	0–1–10
60	3– 8	0–2– 7
60	4– 8	0–3– 6
45	4– 8	0–0–10
35	4– 6	0–0– 7
30	4– 6	0–0– 9
30	5– 7	0–0– 4
20	5– 6	0–0– 8
	Testis (high E)	
23	5– 6	8–0– 0
20	4– 6	0–1– 2
15	4– 6	6–0– 2
15	4– 6	1–3– 5
15	3– 4	0–0– 9
15	4– 6	0–0–12
15	4– 6	0–0–11
15	4– 6	0–0–10
12	4– 6	0–0–11
9	5– 6	0–0– 9
	Epididymis (low E)	
54	5– 8	7–1– 1
40	4– 8	0–0–10
40	4– 8	0–0– 8
30	3– 8	0–0– 4
25	4– 7	0–0– 8
	Epididymis (high E)	
15	4– 7	4–2– 1
15	4– 6	0–0–11
11.5	4– 6	2–2– 4
10	4	1–0– 8
8	5– 6	0–0– 6
	Prost. and S.Ves. (low E)	
60	4– 8	7–0– 1
50	4– 8	0–1– 6
44	3– 6	0–0–10
	Prost. and S.Ves. (high E)	
35	4– 8	6–0– 3
27	5– 7	11–0– 0
20	4– 6	0–0– 9
16	4– 6	0–0–12
	Placenta (high E)	
20	5– 7	7–0– 0
10	5– 6	2–3– 3
10	5– 6	0–0–10
10	5– 6	0–0– 9
6.5	5– 6	0–0– 6
5	5– 6	0–0– 8
	Uterus (high E)	
10	5– 6	8–0– 2
7	5– 6	0–0– 7
6	5– 6	4–0– 2
5	5– 6	0–0– 7
5	5– 6	0–2– 6
	Mammary gl. (low E)	
35	4– 7	4–2– 3
35	4– 6	0–0– 9
30	4– 6	0–0– 9
20	4– 6	0–0–10
10	4– 6	0–0–10
	Mammary gl. (high E)	
10	5– 6	11–0– 0
10	5– 7	8–0– 0
7.7	5– 6	8–0– 0
5	5– 6	12–0– 0
5	5– 6	7–0– 1
5	5– 6	4–2– 0
5	8	2–3– 3
5	6– 7	0–1– 2
3.5	5– 6	9–0– 0
3.5	5– 6	1–2– 4
3.5	4– 6	0–0– 9
2	5– 6	0–0–11
2	5– 6	0–0– 4
	Suckling young (low E)[2] *24–48 hrs.*	
80	3– 8	10–0– 1
80	4– 8	8–0– 1
80	2– 7	6–0– 0
60	4– 7	8–0– 2
60	4– 8	8–0– 0
60	4– 6	7–0– 2
60	4– 6	7–0– 1
45	4– 7	6–1– 2
45	4– 7	0–0–12
45	5– 7	0–0–10
45	4– 6	0–0– 9
45	4– 6	0–0– 8
30	5– 6	0–0–11
30	5– 6	0–0– 9
30	5– 6	0–1– 8
	Brain and Sp. Cord (high E)	
46	3– 7	5–0– 5
40	3– 6	0–0–10
22	4– 7	0–0– 7
12	1– 8	0–0– 9
2.2	6	0–0–10
	Blood (high E)	
80[3]	1–10	0–0– 2
60[3]	1–10	0–0– 9
60[3]	1–10	0–0– 2
40[3]	1–10	0–0–11
40[3]	5– 7	0–0–10
40[3]	3– 5	0–0–10
40[3]	1–10	0–0– 9
36	1– 9	7–1– 0
30	2– 9	0–0–12
30	5– 7	0–0–10
20	2– 7	0–0– 9
13	5	0–0–11
	MASSIVE DOSE OF E[4]	
	Liver	
5.5	5– 6	9–0– 1
4.5	5– 6	3–0– 4
4	5– 6	11–0– 1
2	5	12–0– 0
2	5– 6	10–0– 0
2	5– 6	7–0– 0
1	5	11–0– 0
1	5	9–0– 1
1	5	8–0– 2
1	5	6–0– 2
1	5	3–1– 0
0.6	5	0–1–10
	Muscle	
10	5– 6	10–0– 2
10	5– 6	9–0– 1
10	5– 6	1–1– 8
5	5– 6	9–2– 0
5	5– 6	8–0– 0
5	5– 6	0–0–11
5	5– 6	0–0–11
5	5– 6	0–0– 9
2.5	5– 6	0–0– 8
	Body fat	
7	4– 7	8–0– 0
	Testis and Epid.	
8	5– 6	0–0– 7
8	5– 6	0–0– 4
	Brain	
9.8	5– 6	0–0– 8
	Blood	
19	5– 9	6–0– 3
4	5– 6	0–0– 9

[1] The figures in each line indicate, respectively, the number of grams of fresh tissue fed, the days of pregnancy over which the total dose was divided, and the number of viable fetuses, dead fetuses, and resorption sites observed at the sixteenth day of pregnancy.

[2] Data for twenty-six assays on newborn of low-E and high-E rats, and 24-hour nurslings of high-E rats, have been presented elsewhere (Mason and Bryan, '40, table 2).

[3] These tests were made on an ether-alcohol extract of whole blood, concentrated in vacuo, because of difficulties in feeding large quantities of whole blood. It now seems probable that exposure to light during the process of concentration destroyed much of the vitamin present.

[4] Tissues of "excess-E" rats. The limited supply of animals prohibited more extensive analysis of the tissues. It is estimated that the mean fertility dose of liver and of muscle would be approximately 0.8 and 5 gm., respectively.

period elapsed between consumption of the latter and restoration of the experimental diet. The animals were observed at frequent intervals in order to replace any assay material lost through the screen floor of the cage, and to assure complete consumption of the daily dose.

Vaginal smears were obtained on the fourth, fifth, and tenth to thirteenth days after positive mating. The rats were autopsied or laparotomized at the sixteenth day of pregnancy, and the presence of two or more viable fetuses taken as a criterion of positive response. The latter has been shown to be equivalent to, but much more informative than, the delivery of one or more offspring at term (Mason, '42).

BIO-ASSAY RESULTS

Because of inadequacy of information on the duration of feeding and the individual responses in bio-assays of animal tissues previously reported in the literature, the data obtained have been presented as fully as possible (table 1). Assay tests on certain organs and tissues were unfortunately limited by their small size and by difficulties either in obtaining sufficient amounts for assay purposes or in securing a sufficiently large intake of fresh tissue (e.g., fat) during the first 10 days of pregnancy. The appearance of pseudopregnancy frequently frustrated attempts at assay of valuable material. However, an attempt has been made to estimate the "mean fertility dose" of each tissue tested; that is, the amount of tissue which might be expected to produce a positive response in 50% of a reasonable number of rats tested (as defined by Bacharach, '38). Table 2 presents a summary of these estimates, based upon the 312 assay tests of table 1 supplemented by twenty-six others previously reported (Mason and Bryan, '40). When due allowance is made for animal variations in response to critical levels of dosage and for the small gradations in dosage levels used, in comparison with those generally employed in bio-assay tests, many of these estimates seem equitable. Certain others, indicated in table 2 by a question mark, must be regarded as conjectures. Since the

method of preparation of virgin rats for assay tests unequivocally excludes the possible occurrence of "first litter fertility" (Mason and Bryan, '38, '40; Mason, '42), the responses recorded in table 1 can be accepted with confidence.

TABLE 2

The estimated "mean fertility dose" of tissues from low-E and high-E rats, expressed as grams of fresh tissue, and the calculated difference in concentration of the vitamin.

TISSUE	LOW-E RATS GM. OF TISSUE	HIGH-E RATS GM. OF TISSUE	DIFFERENCES IN E-CONTENT (TIMES)
Liver	120	8.5	14.1
Muscle	60	15	4.0
Kidney	60	13	4.6
Body fat	50	17	2.9
Pancreas and thymus	45 ?	10 ?	4.5
Heart	35	9	3.9
Lung	35	8	4.4
Spleen	30	10 ?	3.0 ?
Testis	60	20 ?	3.0 ?
Epididymis	55 ?	15 ?	3.7 ?
Prostate and sem. ves.	60 ?	20 ?	3.0 ?
Placenta	—	13 ?	—
Uterus	—	8 ?	—
Mammary gland	35 ?	4	8.7 ?
Newborn	200 [1]	50 [1]	4.0
Suckling young (24–48 hours)	50	15 [1]	3.3
Brain and spinal cord	—	50 ?	—
Blood (whole)	—	40 ?	—

[1] Based upon assay data presented in a previous report (Mason and Bryan, '40; table 2).

Several interesting facts are apparent from these data. (1) Substances possessing the biological activity of vitamin E are widely distributed throughout the tissues and organs of the rat; the concentration of the vitamin in those tissues which undergo pathological alteration after E-deprivation (e.g., fetus, testis, musculature, uterus, brain and spinal cord) differs in no significant manner from that in other tissues. This stands in striking contrast to what is known concerning the distribution of vitamin A in the rat (Popper and Greenberg, '41).

(2) At low levels of intake the liver contains about one-half as much vitamin E per gram of fresh tissue as the skeletal muscles, kidney, body fat, testis, epididymis, prostate and seminal vesicle, and about one-fourth as much as the heart, lung and spleen. Apparently, the metabolic needs of body tissues of low-E rats are satisfied at the expense of liver storage, suggesting that four times the minimal daily requirement is considerably below the optimal requirement. This would be in full accord with what is known concerning other vitamins.

(3) Increasing the daily intake from four to 100 times the minimal requirement permits a fourteenfold increment in liver storage but raises that in the other tissues only three to four and one-half times. At an intake approximately 10,000 times the minimal (see table 1), the storage in liver and muscle increases to 150 and to twelve times, respectively, that in low-E rats.

(4) In both low-E and high-E rats the active mammary gland possesses a greater storage capacity for E than the liver. In view of observations that mammary transfer is decidedly limited (Mason and Bryan, '40), it would appear that concentration of the vitamin in the glandular tissue greatly exceeds that in the secretion produced.

(5) The E-content of newborn rats is much less than that of the adult tissues examined[5] and is augmented to the same degree as the latter (exclusive of the liver and mammary gland) by increased intake of the vitamin. A fourfold increment occurs in offspring of both low-E and high-E rats after 24–48 hours lactation, bringing the E-content up to that of adult muscle, body fat and kidney. It cannot be stated, however, what proportion of the vitamin existed in the stomach contents and what proportion was actually stored in the tissues of the young rat.

[5] The few data available suggest that the vitamin E content of nervous tissue and of whole blood more closely resembles that of the newborn than that of other adult tissues.

DISCUSSION

These studies confirm the conclusions of Evans and Burr relative to the wide distribution of vitamin E in the body of the rat but indicate that musculature and body fat, which they considered to possess the greatest concentration of the vitamin, actually contain an amount similar to or less than that present in other tissues and organs, whether the previous intake be low or high. Their low values for the vitamin in rat liver, which they recognized as being incompatible with the remarkable storage capacity of this organ for other fat soluble vitamins, may be explained by the fact that their donor rats received a sub-optimal intake of vitamin E not unlike that of the low-E rats used in the present study. Although the liver constitutes the chief repository for vitamin E when the intake is optimal or greater, its storage capacity seems to be decidedly less than that for vitamins A and D. It is obvious, however, that its degree of saturation represents the most reliable index of previous intake and storage. Bio-assays of human and monkey livers by Mr. R. Terry in this laboratory (unpublished studies) have demonstrated a storage about intermediate between that observed in low-E and high-E rats. On the other hand, livers from dogs with bile fistulae have invariably given negative responses at similar and higher levels of feeding, which confirms earlier observations of Greaves and Schmidt ('37) concerning the failure of bile-fistula rats to absorb vitamin E.

It cannot yet be stated what significance may be attached to the disparity between the concentration of the vitamin in newborn rats and that in the uterus and placenta, or between that in nurslings and in the mammary gland. No adequate explanation can be offered for the exceptionally high content of E in the latter tissue.

The small proportion of ingested vitamin which is stored by the rat, and the fate of the remainder, constitute problems warranting further investigation. Assuming that the various tissues tested in the present studies would constitute one-half

the weight of the rat, and assuming that the remaining tissues possessed an equivalent amount of the vitamin, it is estimated that the total storage in low-E and high-E rats of 300 gm. body weight would amount to about three and fourteen mean fertility doses of vitamin E, respectively. In the case of the high-E rats, this would represent only about three times the daily intake. In confirmation of the conclusions of Moore and Rajagopal ('40) and of Cuthbertson et al. ('40) based upon spectroscopic estimation of E in the tissues, urine and feces of the rat, it appears that the tocopherol molecule is either readily broken down by the body or is ineffectively absorbed and stored.

SUMMARY AND CONCLUSIONS

1. A total of 338 bio-assay tests on tissues of rats receiving approximately 4, 100 and 10,000 times the minimal daily requirement of vitamin E reaffirms its wide distribution in the animal body.

2. At the low level of intake, which is considered sub-optimal, the heart, lung and spleen possess almost twice as much vitamin E per gram of fresh tissue as the musculature, body fat and other visceral organs (kidney, testis, epididymis, prostate and seminal vesicle), and about four times as much as the liver.

3. At moderately high levels of intake the storage in the viscera, musculature and body fat is augmented three to four and one-half times; the heart, lung and spleen receiving more than other tissues, while that in the liver is increased about fourteen times. The mammary gland concentrates about twice as much E as the liver.

4. At excessively high levels of intake the musculature and liver, respectively, possess about 12 and 150 times the vitamin E storage occurring at the low level of intake. Liver content of the vitamin affords the most useful measure of the previous intake and storage.

5. Despite the wide distribution of vitamin E in the body of the rat, the estimated total storage represents but a small fraction of the vitamin ingested, confirming the suggestion of others that the tocopherol molecule is either ineffectively absorbed or readily broken down in the organism.

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HEAT PRODUCTION OF THE RABBIT AT 28°C. AS AFFECTED BY PREVIOUS ADAPTATION TO TEMPERATURES BETWEEN 10° AND 31°C.[1]

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Several investigators have shown that the level of the heat production of the rat and of other small animals measured at a given environmental temperature is dependent on the temperature at which these animals were previously living. Kayser ('39), Ring ('39), Gelineo ('35), Giaja and Gelineo ('31), and Schwabe and his colleagues ('38) have noted differences in the basal metabolic rates of rats according to the different temperature levels at which they had been living before measurement, and in some cases this difference amounted to over 20%. An influence of adaptation to previous environmental temperature has also been found with small birds by Kayser ('29), Dontcheff and Kayser ('34), Gelineo ('34), and others. It is of interest to determine whether this effect of adaptation is confined to small animals or is true also of larger animals. During an investigation of the basal metabolic rate of rabbits and the factors affecting this rate, it became evident that the environmental temperature at which the rabbits had previously been living influenced their metabolism, despite a 24-hour period of adjustment at thermic neutrality prior to measurement. Hence a study was made to determine how the rabbit's metabolism measured at 28° is affected by the previous environmental temperature and by the length of stay at that temperature.

[1] A preliminary report of this study was presented on April 18, 1941, at the Chicago meeting of the American Physiological Society. (Am. J. Physiol., vol. 133, p. P360, 1941.)

PROCEDURE

Twelve adult rabbits of both sexes were used, including Dutch, Chinchilla, and New Zealand White breeds ranging in weight from 1.9 to 5.3 kg. Group I consisted of five rabbits whose average weight was 3.99 kg., and group II of seven other rabbits whose average weight was 3.24 kg. The average weight of the animals in each group did not change significantly throughout the experimental period.

An open-circuit system, employing the Carpenter gas-analysis apparatus, was used for the metabolic measurements,[2] as in our previous work on rabbits (Lee, '39 a). The first two measurements on any rabbit have been discarded, because the rabbit had not become adjusted to the experimental procedure. Only periods of complete repose have been used.

The animals lived for a stated period at a definite temperature level between 10° and 31°C. The average variation in daily temperature during this period did not exceed 3°C. at any environment except as noted for group I, series 5. The rabbit was then kept at 28° for 24 hours, without food, and immediately thereafter its metabolism was measured at 28° to 29°C. To establish the effects of living at these several temperatures on the metabolism measured at 28°, from three to five experiments were carried out with each rabbit following each period of adaptation. The rabbits in group I, in all the series except no. 2, had been living at a definite temperature for 1 week or more before measurements were made. Those in group II in all instances lived at the given temperature level for 3 to 4 weeks before the measurements. In each group the series were consecutive. The length of each series, as recorded in table 1, includes the period of adaptation and the period of metabolism measurements.

The results were calculated as total heat production per 24 hours, but because of differences in the individual weights of the rabbits from series to series, it was not considered correct to use these measured total heat values to estimate

[2] The author wishes to acknowledge the technical assistance of Mr. George Lee.

the effect of previous environmental temperature on the metabolism. Instead, the predicted basal total heat production of each rabbit was calculated from its average weight in each series by using the formula:

$$\text{Total heat production per 24 hours} = 40W + 20$$

in which W represents the weight in kilograms (Lee, '39 b). Calculation was then made of the percentage deviation of each rabbit's measured total heat production in each series from its predicted basal heat production for that series. These individual percentage deviations in each series were then compared with the percentage deviations from prediction noted in series 3, for with both groups the measurements in series 3 were representative of the basal metabolism. The percentage deviation from prediction found for each rabbit in series 3 was therefore used as a zero point to calculate, in the other series, the total percentage change in metabolism from the basal level. The individual total percentage changes for the several rabbits in each series were then averaged. It is believed that this treatment of the data corrects for any role that might have been played by the small individual differences in body weight. An example of the method of calculation follows.

Rabbit no. R-1 (group II)

SERIES NO.	WEIGHT	TOTAL HEAT PRODUCTION PER 24 HOURS, CAL.		CHANGE FROM PREDICTION
		Measured	Predicted basal	
	kg.			%
1	2.14	121.7	105.6	+15.3
3	2.47	112.7	118.8	— 5.1

$$\text{True change from basal level} = \frac{115.3}{94.9} = +21.5\%.$$

DISCUSSION OF RESULTS

In group I the rabbits first lived at 17° for 7 weeks (series 1, table 1), during the last four of which they were measured weekly. Under these conditions the heat production of the individual rabbits, measured at 28°, was respectively, 11.6, 20.2, 35.1, 17.1, and 11.1% above their baseline levels in series

3 and averaged 19.0% above the baseline. The third and fifth rabbits in this group had basal metabolic rates significantly below the prediction, yet their reactions to changes in environmental temperature were similar to those of the other rabbits.

Following series 1 the rabbits were shifted to an environment of 29° and measured on the second, fourth, seventh, and fourteenth days of stay at this temperature (series 2). The levels on the fourteenth day averaged 1.8% above the baseline.

TABLE 1

Metabolism of rabbits in group I, measured at 28°C.

SERIES NO.	LENGTH OF SERIES	PREVIOUS TEMPERATURE (AVERAGE)	MEASUREMENT	PER CENT CHANGE FROM BASELINE IN SERIES 3[1]
	weeks	°C.		
1	7	17	Average	+19.0
2	2	29	Average	+ 5.5
			14th day	+ 1.8
3	3	29	Average	± 0.0
			30th day at 29°	— 2.2
4	4	29	Average	— 4.3
			9th week at 29°	— 5.5
5	3	29	Average	— 1.6
			(except 2 hours daily at 17°C.)	
6	3	18	Average	+ 4.0
7	4	23	Average	+ 0.4
8	4	25	Average	— 1.5
9	7	23	None	—
10	7	28	Average	— 1.7
11	3	31	Average	— 7.1
12	9	22	Average	+ 3.1
			Metabolism of rabbits in group II, measured at 28°C.	
1	9	10	Average	+17.0
2	6	22	Average	+ 5.3
3	8	29	4th week	+ 0.8
			Average	± 0.0
			8th week	— 1.5
4	7	23	Average	+ 4.2

[1] See explanation on page 85.

The measurements during the next 3 weeks (series 3) were obtained under conditions that conform to the prerequisites for basal metabolism measurements (Lee, '39 a). The percentage changes from baseline on the thirtieth day at 29° (series 3) were slightly lower than the average for the entire series, indicating that the heat production was still decreasing, although the major change occurred in the first 2 weeks. A further small decrease occurred on keeping the animals at 29° for another 4 weeks (series 4), and the values for the last week (ninth week at 29°) were slightly below the average for the series, showing that the animals were still adjusting themselves to the temperature.

To determine the effect of increasing the daily range of environmental temperature, the rabbits, still maintained at 29°, were placed for 2 hours each day at 17°C. over a period of 3 weeks (series 5). Under these conditions the heat production was slightly increased above that in the previous series. Next the group was placed at 18° for 3 weeks, with the result that the metabolism was noticeably increased. It did not, however, approach the initial level at 17°, for the stay at 18° following the long period of adaptation at 29° was too short to permit complete readjustment to the former level.

The temperature was then raised in small steps (series 7 to 11). This produced a consistent decrease in metabolism except in rabbits in series 10, which was preceded by a 7-week period (series 9) in which the temperature averaged 2° lower than in the previous series. There were no measurements during this period. The increase in temperature in series 10 resulted in a metabolic level close to that in series 8.

The minimum metabolism was found when the rabbits lived at 31° for a 3-week period (series 11), after a long, gradual approach to this temperature. This minimum level was 7.1% below the baseline in series 3. In series 12 the rabbits were kept at 22°, and the heat production increased markedly.

The differences in the temperatures at which the rabbits lived before measurement (17° to 31°C.) caused the heat

production, always measured at 28°, to show a maximum range of 26.1% in the deviations from the baseline.

It appears that the adjustment of the 28° metabolism to an increase in temperature took place largely in a short period of time, whereas the adjustment to a decrease in temperature did not take place so rapidly.

Further experiments in later series were complicated both by reduction in the number of rabbits in group I and by other features. The general trends indicated by these experiments were that when the temperature was maintained at 15° for 8 weeks, the heat production increased markedly, and that prolongation of the stay at 15° for 5 weeks more resulted in a further increase in heat production, which approached the initial level at 17°C. When, in the final series, the temperature was raised to 23°, the metabolic level decreased again.

The same trends of metabolic reaction to previous environmental temperature were shown by group II (table 1). The rabbits in this group were adapted initially to 10° for a period of 9 weeks. Here again as the temperature of the environment was increased, the metabolism measured at 28° decreased. The first measurements in series 3 were made after the rabbits had been at 29° for 4 weeks, and these measurements averaged 0.8% above the average baseline for this series. Measured in the eighth week at this temperature, the metabolic level was 1.5% below the average baseline. This comparison of the initial and final measurements in the same series after an interval of 4 weeks brings out the same picture as shown by group I, namely, that there is a further decrease in heat production as the stay at thermic neutrality is prolonged, at least up to 8 weeks. When the previous temperature was lowered again (series 4), the metabolic level increased. The maximum changes found in metabolic level were, for the individual rabbits, 29.9, 21.5, 7.5, 15.6, 17.8, 15.9, and 11.0% above the baseline and averaged +17%.

In 89% of eighty cases in both groups in which the previous temperature was changed from one level to another or was maintained for a longer period at the same level, it was noted,

respectively, that the metabolism varied inversely with the direction of the change in temperature or additional change in metabolism took place in the direction previously noted.

The maximum differences found in groups I and II between the heat production resulting from adaptation to temperatures of 17° and 29° and of 10° and 29°, respectively, are in line with the difference noted by Ring with rats whose metabolism, measured at thermic neutrality after they had lived at 3°, was increased 21% above the basal metabolism when they had been at 30°C. (thermic neutrality).

In an earlier paper describing the prerequisite conditions for measurement of the basal metabolism of the adult rabbit, it was stated that "the rabbits should have been living at environmental temperatures above 20° but not above 32°." Many of the measurements in both group I and group II were made under these requirements for basal metabolism measurements, and except for series 11, group I, the values are within ±5.5% of the average series value taken as the baseline. Even within the limits of 20° to 32°C. the metabolic level can be measurably influenced by changes in previous environmental temperature. Certainly in studies in which changes in basal metabolism are used as measures of the effects of superimposed conditions, the environmental temperature should be maintained at a given level between 20° and 32°C., preferably 28° to 29°, and the rabbit should be habituated to the temperature for 3 weeks prior to the measurements. The effect of previous environmental temperature offers a partial explanation of the wide variations noted in the literature in the so-called "basal metabolism measurements" on rabbits.

SUMMARY

The level of the metabolism of twelve adult rabbits, in repose, at 28°, measured after they had been kept at 28° and without food for 24 hours, was dependent on the temperature at which they had been living prior to this period and the length of time they had been at this temperature. The major metabolic adjustment to an increase in temperature toward

thermic neutrality took place in 2 to 3 weeks, but further adjustment continued up to at least 2 months. The adjustment to lower temperatures was less rapid. The maximum range in the deviations from the basal level in the metabolism measured at 28° amounted to 26.1% in a group of five rabbits that had lived at 17° and subsequently at 31°, and to 17.0% in a group of seven rabbits that had lived at 10° and later at 29°C. In general, the metabolism varied inversely with the change in previous temperature, 89% of eighty observations conforming to this finding. Prolongation of the stay at a given temperature caused a further change in metabolism in the same direction. When basal metabolism measurements on the rabbit are to be used as a measure of the effect of a superimposed condition, the previous environmental temperature should be maintained at 28° to 29°C., and the rabbit should be habituated to this temperature for 3 weeks prior to the measurements.

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CHOLINE METABOLISM

VIII. THE RELATION OF CYSTINE AND OF METHIONINE TO THE REQUIREMENT OF CHOLINE IN YOUNG RATS [1]

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FOUR FIGURES

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A supplement of cystine in a choline-deficient diet increases both the deposition of liver fat in older rats (Beeston and Channon, '36) and the incidence and severity of hemorrhagic degeneration in young rats (Griffith and Wade, '40). Griffith ('41) found no direct relation between the level of the cystine supplement and the degree of the cystine effect and suggested that the observed increase in the requirement of choline was due to an improved nutritional state rather than to some direct antagonism of cystine and choline. Although cystine was recognized as the limiting essential amino acid in casein following the classical experiments of Osborne and Mendel ('15), emphasis was shifted to the indispensability of methionine rather than of cystine by the observations of Womack, Kemmerer and Rose ('37). However, Rose ('37) pointed out that supplementary cystine greatly improves a diet which contains insufficient methionine for the total sulphur requirement. Rations containing casein as the protein are occasionally fortified with cystine (Mackenzie et al., '39) but this practice has not been general. The present paper describes experiments which are interpreted as demonstrating

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that the 18% casein diet is a suboptimal source of cystine and that supplementary cystine improves the nutritional level and thereby indirectly increases the requirement of choline. Evidence is also presented for the conclusion that methionine which serves as a source of labile methyl (du Vigneaud et al., '40; Griffith and Mulford, '41) does not serve as a source of cystine sulphur.

EXPERIMENTAL

Young male rats, 21 to 26 days of age and 38 to 42 gm. in weight, were used and were equally distributed in the various groups according to age. The appearance of the kidneys was noted at the end of the experimental period and the per cent of animals in each group showing the hemorrhagic state (Griffith, '40) is recorded in figures 2, 3 and 4. The term liver fat in figures 1 to 4 refers to the total chloroform-soluble substances of the liver. The distance between the tip of the nose and the anus was measured and is recorded in figure 1 and in tables 1 and 2 as body length.

The low choline diet, AC 50, consisted of purified casein 18%, lard 19, sucrose 49, salt mixture 4 (Hawk and Oser, '31), calcium carbonate 1, agar 2, powdered yeast 6 [2] and fortified cod liver oil 1%. All supplements of choline and cystine and other additions to the basal diet replaced an equal weight of sucrose.

RESULTS AND DISCUSSION

Effect of a supplement of cystine in an 18% casein diet. The effect of the addition of cystine to diet AC 50 plus various levels of choline during 20- or 40-day periods is shown in figure 1 and in tables 1 and 2. In every case, the rats receiving the extra cystine were heavier and longer and these gains were accomplished with little or no increase in the consumption of food, the gain in weight per gram of food during a 20-day period averaging 0.55 gm. on the basal diet and 0.60 to 0.62 gm. on the supplemented diet. The more

[2] Anheuser-Busch, strain G.

efficient utilization of food is evident during the first but not during the second 20-day period (table 1), a finding which suggests a greater need of cystine in young rats 3 to 6 or 7 weeks of age. The beneficial effect of the extra cystine is emphasized by the difference in the weights of groups 2 and 6 (table 1) after 150 days on the basal and supplemented diets (data not shown in table 1). At the end of this period the rats in group 2 averaged 286 gm. in weight, seven weighing

TABLE 1

The effect of supplements of cystine in an 18% casein diet (AC 50) on growth and utilization of food in 40-gm. male rats during a 40-day experimental period.

GROUP	1	2	3	4	5	6	7	8[1]
No. of rats in group	40	20	20	20	20	20	38	20
Dietary casein, %	18	18	18	18	18	18	18	15
Cystine supplement, %	0	0	0.04	0.08	0.3	0.5	0.3	0.31
Choline chloride supplement,%	0.5	0.3	0.3	0.3	0.3	0.3	0.5	0.3
Gain in weight, gm.								
20 days	77	79	88	93	87	91	84	91
40 days	155	158	174	177	172	176	165	177
Body length, cm.								
20 days	17.7	17.8	18.1	18.4	18.2	18.4	18.2	18.4
40 days	20.8	21.2	21.2	21.4	21.5	21.8	21.2	21.8
Gain in weight per gram of food, gm.								
0 to 20 days	0.56	0.54	0.56	0.59	0.64	0.63	0.61	0.60
0 to 40 days	0.46	0.46	0.47	0.47	0.50	0.49	0.47	0.49
20 to 40 days	0.39	0.39	0.40	0.39	0.41	0.40	0.38	0.40

[1] 0.09% dl-methionine added to diet.

less than 270 gm. and five weighing more than 300 gm.; in contrast, the rats in group 6 averaged 312 gm. in weight, none weighing less than 270 gm. and eleven weighing more than 300 gm.

A 15% level of casein, supplemented with both methionine and cystine, permitted normal growth during a 40-day period (group 8, table 1) and methionine alone satisfactorily replaced the cystine supplement in the 18% casein diet during a

20-day period (group 6, table 2). The addition to the basal diet of 4% of lactalbumin (group 8, table 2) also satisfied the requirement of sulphur amino acids but the 22% casein diet appeared less efficient (group 7, table 2). Growth in these experiments was not limited by a deficiency in vitamins of the B complex because no additional improvement followed an increase in the level of yeast from 6% to 10% (group 9, table 2). All of the rats listed in table 2 received a supplement of 5 mg. of α-tocopherol on the tenth day. The results

TABLE 2

The effect of supplements of cystine, methionine, yeast and protein in an 18% casein diet (AC 50 + 0.3% choline chloride) on growth and utilization of food in 40-gm. male rats during a 20-day experimental period.

GROUP	1	2	3	4	5	6[1]	7[2]	8[3]	9[4]
No. of rats in group	24	23	24	23	23	24	22	24	22
Cystine supplement, %	0	0.1	0.2	0.3	0.5	0	0	0	0
Gain in weight, gm.	71	77	80	80	80	81	75	81	83
Body length, cm.	17.6	17.9	18.2	18.2	18.2	18.2	17.9	18.2	18.2
Gain in weight per gram of food, gm.	0.55	0.58	0.62	0.62	0.63	0.61	0.58	0.60	0.60
Dietary sulphur, %[5]	0.134	0.161	0.188	0.214	0.269	0.214	0.164	0.190	0.214

[1] 0.362% dl-methionine added to diet.

[2] 4% casein added to diet.

[3] 4% lactalbumin added to diet.

[4] 4% yeast added to diet.

[5] Calculated from analyses reported by Baernstein ('36). Sulphur of yeast not included in total.

with groups 3 and 4 (table 1) and with groups 2 and 3 (table 2) indicate that the 18% casein diet requires 0.1 to 0.2% of additional cystine to permit optimum growth and utilization of food.

Relation of dietary cystine and choline to choline deficiency. The effect of cystine and of choline on liver fat is illustrated in figure 1 which shows the increase in liver fat due to a cystine supplement in low choline diets during a 20-day period. An adequate level of choline prevents this action of cystine

but does not influence appreciably its effect on growth and on utilization of food. Figure 2 shows the effect of three levels of choline and of six levels of cystine on liver fat and on renal lesions during an 8-day period. The deposition of liver

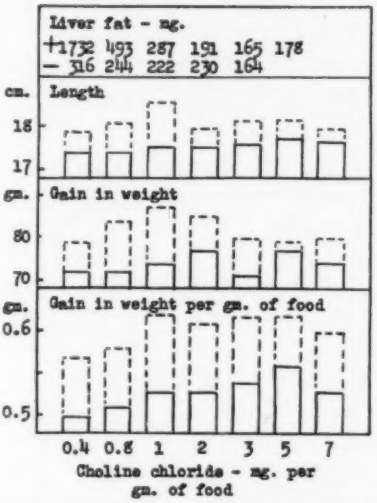

Fig. 1 Relation of supplementary choline and cystine (0.3%) in an 18% casein diet (AC 50) to the deposition of liver fat, to growth and to the utilization of food in 40-gm. male rats during a 20-day period. The groups receiving cystine are indicated by the broken lines. Each group consisted of twenty or more rats.

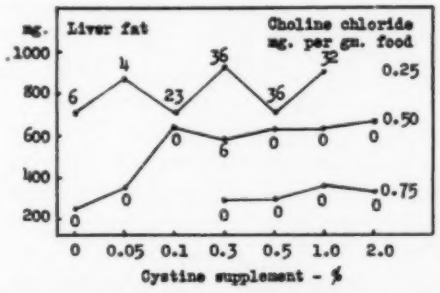

Fig. 2 The relation of the level of a supplement of cystine to the deposition of liver fat and to the incidence of renal lesions in 40-gm. male rats during an 8-day period. The numbers on the curves show the per cent of animals with renal lesions. Each group consisted of twenty or more rats.

fat was variable with 0.25 mg. of choline per gram of food, a result which is explained by the poor nutritional state of such animals (Griffith and Wade, '40). Groups fed rations containing 0.5 to 0.75 mg. of choline per gram of food showed no effect of the level of the cystine supplement provided 0.1% or more of cystine was added. These results extend and confirm the previous observation (Griffith, '41) that the minimum daily requirement of choline for the protection of the kidneys is the same on diets containing 0.3% or 1.0% of added cystine.

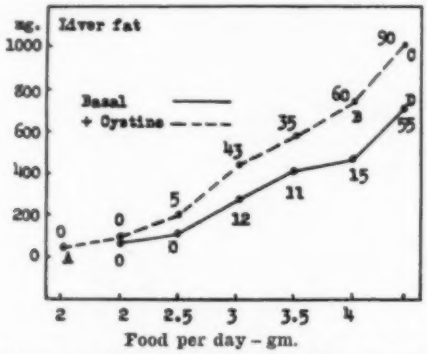

Fig. 3 The relation of restriction of the consumption of diet AC 50, with and without a supplement of 0.5% of cystine, on the deposition of liver fat and on the incidence of renal lesions in 40-gm. male rats during an 8-day period. The numbers on the curves show the per cent of animals with renal lesions. Group A received a special diet which contained in 2 gm. the same amount of each constituent except lard and sucrose that was present in 4 gm. of AC 50 + 0.5% cystine (group B). Groups C and D consumed the experimental diets ad libitum, the average daily food intakes being 4.6 and 4.9 gm., respectively. Each group consisted of twenty or more rats.

It is significant that a supplement of 0.1% of cystine in the 18% casein diet produced the maximum deposition of liver fat as well as nearly optimum growth and utilization of food (tables 1 and 2).

Griffith and Mulford ('41) found that the choline requirement is decreased by restriction of the intake of a food which is injurious if fed ad libitum. The comparison in figure 3 of the effect of restriction of the basal diet, with and without

a supplement of cystine, demonstrates that the signs of choline deficiency are increased both by the cystine supplement and by the ingestion of larger quantities of food. Of particular interest is the result with group A which was fed 2 gm. daily of a food mixture containing the same amount of each dietary constituent, except lard and sucrose, that was present in 4 gm. of the food supplied group B. Group A consumed 0.5 gm. of lard and 0.2 gm. of sucrose and group B consumed 0.8 gm. of lard and 1.94 gm. of sucrose daily. Therefore, group A, with no signs of choline deficiency, and group B, with a high incidence of renal lesions and with markedly fatty livers, consumed rations differing mainly in caloric value.

It seems logical to conclude that the increased need of choline in young rats on an 18% casein diet supplemented with cystine is due to the stimulation of metabolism resulting from the addition of required cystine to a cystine-deficient food mixture. No explanation is thus necessary for the failure of excess cystine to affect the choline requirement. There is no evidence in these experiments that cystine is toxic per se. It is entirely possible that the administration of an excess of cystine may result in injury to the tissues of the rat (Curtis et al., '27; Lillie, '32; Earle and Victor, '41). However, the present study suggests that due consideration be given the character of a food mixture before it is concluded that an effect of a cystine supplement is directly related to the cystine rather than to a stimulation of metabolism which in turn brings to light previously unrecognized deficiencies. On the other hand it is pertinent that a supplement of cystine offsets the toxicity of certain injurious compounds (White and Jackson, '35; Stekol, '37; White and White, '39) and halves the time required for the action of an anti-gray hair factor (Pavcek and Baum, '41).

Relation of dietary cystine and casein to choline deficiency. It became of interest to determine at which level of dietary casein the effect of the cystine supplement is no longer evident. Figure 4 shows that in the absence of choline 30% of casein is required to supply enough methionine to prevent signs of

choline deficiency. This is also the level at which the effect of added cystine disappears. In other words, a diet containing 26% of casein appears deficient in both methionine (labile methyl) and in cystine in spite of the fact that this diet contains as much total sulphur (0.194%) in the form of methionine and cystine as is present in the adequate choline-supplemented 18% casein diets to which additional cystine or lactalbumin is added (groups 3 and 8, table 2). It is therefore concluded that, if methionine is used as a source of methyl for the

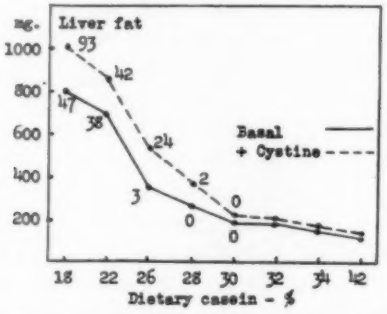

Fig. 4 The relation of supplementary cystine (0.3%) and of dietary casein (18 to 42%) to the deposition of liver fat and to the incidence of renal lesions in 40-gm. male rats during an 8-day period. Casein above 18% replaced an equal weight of sucrose in the basal diet, AC 50. The numbers on the curves show the per cent of animals with renal lesions. Each group consisted of twenty or more rats.

synthesis of choline, its sulphur is not available for the formation of cystine. Such a conclusion is not compatible with the demethylation of methionine to labile methyl and homocysteine if a direct conversion of homocystine to cystine occurs (du Vigneaud et al., '33). It is in agreement with the idea expressed by Toennies ('40) that homocysteine must first be converted to methionine before its sulphur is used as cystine sulphur. This conversion would not be possible on the 26% casein diet, unless supplementary choline or some other source of labile methyl is added, because this diet is deficient in labile methyl.

SUMMARY

1. Supplementary cystine (0.1 to 0.2%) or methionine improves the growth and efficiency of utilization of food in young rats on an 18% casein diet.

2. The deposition of liver fat and the incidence of renal hemorrhagic degeneration in young rats on a low choline diet are increased by supplements of cystine (0.1 to 0.2%) which improve the nutritive value of the diet. Larger supplements of cystine have no effect on the requirement of choline.

3. In the absence of dietary choline, 30% of casein is required to supply sufficient methionine and cystine for the prevention of signs of choline and cystine deficiency.

4. Evidence is presented for the conclusion that methionine which is needed and used as a source of labile methyl is not utilizable as a source of cystine sulphur.

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